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(54) **Title:** FCγ POLYMORPHISMS FOR PREDICTING DISEASE AND TREATMENT OUTCOME

(57) **Abstract:** The invention provides compositions and methods for determining the likelihood of successful treatment with Cetuximab or other equivalent. The methods comprise determining the genomic polymorphism present in a predetermined region of the FcγRIIa gene at amino acid position 131 and/or alternatively the FcγRIIIa gene at amino acid position 158.

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FCγ POLYMORPHISMS FOR PREDICTING DISEASE AND TREATMENT OUTCOME

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority under 35 U.S.C. § 119(e) to U.S. Application Serial Nos. 60/741,405 and 60/779,218, filed November 30, 2005 and March 3, 2006, respectively, the contents of each of which is incorporated herein in its entirety.

FIELD OF THE INVENTION

This invention relates to the field of pharmacogenomics and specifically to the application of genetic polymorphism(s) to diagnose and treat diseases.

BACKGROUND OF THE INVENTION

In nature, organisms of the same species usually differ from each other in some aspects, e.g., their appearance. The differences are genetically determined and are referred to as polymorphism. Genetic polymorphism is the occurrence in a population of two or more genetically determined alternative phenotypes due to different alleles. Polymorphism can be observed at the level of the whole individual (phenotype), in variant forms of proteins and blood group substances (biochemical polymorphism), morphological features of chromosomes (chromosomal polymorphism) or at the level of DNA in differences of nucleotides (DNA polymorphism).

Polymorphism also plays a role in determining differences in an individual's response to drugs. Pharmacogenetics and pharmacogenomics are multidiscinplinary research efforts to study the relationship between genotype, gene expression profiles, and phenotype, as expressed in variability between individuals in response to or toxicity from drugs. Indeed, it is now known that cancer chemotherapy is limited by the predisposition of specific populations to drug toxicity or poor drug response. For a review of the use of germline polymorphisms in clinical oncology, see Lenz, H.-J. (2004) J. Clin. Oncol. **22(13)**:2519-

2521; Park, D.J. et al. (2006) Curr. Opin. Pharma. **6(4)**:337-344; Zhang, W. et al. (2006) Pharma. and Genomics **16(7)**:475-483 and U.S. Patent Publ. No. 2006/0115827. For a review of pharmacogenetic and pharmacogenomics in therapeutic antibody development for the treatment of cancer, see Yan and Beckman (2005) Biotechniqes **39**:565-568.

Colorectal cancer (CRC) represents the second leading lethal malignancy in the USA. In 2005, an estimated 145,290 new cases will be diagnosed and 56,290 deaths will occur. Jemal, A. et al. (2005) Cancer J. Clin. **55**:10-30. Despite advances in the treatment of colorectal cancer, the five year survival rate for metastatic colon cancer is still low, with a median survival of 18-21 months. Douglass, H.O. et al. (1986) N. Eng. J. Med. **315**:1294-1295.

The Food and Drug Administration has approved the use of Cetuximab, an antibody to the epidermal growth factor receptor (EGFR), either alone or in combination with irinotecan (also known as CPT-11 or Camptosar®) to treat patients with EGFR-expressing, metastatic CRC, who are either refractory or intolerant to irinotecan-based chemotherapy. One recent study (Zhang, W. et al. (2006) Pharmocogenetics and Genomics **16**:475-483) investigated whether polymorphisms in genes of the EGFR signaling pathway are associated with clinical outcome in CRC patients treated with single-agent Cetuximab. The study reported that the cyclin D1 (CCND1) A870G and the EGF A61G polymorphisms may be useful molecular markers for predicting clinical outcome in CRC patients treated with Cetuximab.

Other polymorphisms have been reported to associated with clinical outcome. Twenty-one (21) polymorphisms in 18 genes involved in the critical pathways of cancer progression (i.e., drug metabolism, tumor microenvironment, cell cycle regulation, and DNA repair) were investigated to determine if they will predict the risk of tumor recurrence in rectal cancer patients treated with chemoradiation. Gordon, M.A. et al. (2006) Pharmacogenomics **7(1)**:67-88.

A single nucleotide polymorphism of the FCγIIIA gene results in two allotypes of Fcγ receptor IIIA with valine (V) or phenylalanine (F) at amino acid 158. This and additional Fcγ polymorphisms have been used to predict susceptibility to autoimmune disease (Bottcher, S. et al. (2005) J. Immunol. Methods **306(1-2)**:128-136; Hirankarn, N. et al.

(2006) Tissue Antigens **68(5)**:399-406; Lorenz, H-M. et al. (2006) Aktuelle Rheumatologie **31(1)**:48-55 and Chen Y-Y, et al. (2006) Clin. and Exper. Immunol. **144(1)**:10-16 and U.S. Patent Publ. Nos. 2006/0099633) to responsiveness to antineoplastic therapy (U.S. Patent Publ. No. 2006/0008825), responsiveness to interleukin-2 therapy (U.S. Patent Publ. No. 2006/0165653 and 2006/0008825, PCT Publ. No. 2006/002930) and peridontal status (Wolf, D.L. et al. (2006) J. Clin. Peridontology **33(10)**:691-698). However, to the best of Applicant's knowledge, polymorphisms in the FCγ gene have not heretofore been reported to correlate with clinical outcome in CRC patients treated with Cetuximab.

DESCRIPTION OF THE EMBODIMENTS

Two primary mechanisms are responsible for the cytotoxic activity of monoclonal antibodies: antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxcicity (CDC). Carter, P. (2001) Nat. Rev. Cancer **1**:118-129. Tumor cell killing by ADCC is triggered by the binding of the Fc region of an antibody to cell surface immunoglobulin G γ (IgG γ) Fc receptors on immune effector cells, including macrophages, monocytes, dendritic cells, natural killer (NK) cells, and neutrophils. CDC is initiated by complement component C1q binding to the Fc region of an antibody when bound to the surface of a tumor cell. Subsequent target-cell lysis can occur in a cell-dependent or cell-independent manner. Carter, et al. (2001) supra.

FcγRs are of two main types: activating (e.g. the high affinity receptor CD64 (Fcγ RI)) and the low affinity receptors CD32A (FcγRIIa) and CD16A (FcγRIIIA) or the inhibiting (FcγRIIB). Carter, et al. (2001) supra; Clynes, et al. (2000) PNAS **95**:652-656; Cartron, G. et al. (2002) Blood **90**:754-758; Van de Winkel et al. (1993) Immunol. Today **14**:215-221; Kumpel, B.M. et al. (2003) Clin. Exp. Immunol. **132**:81-86; Warmerdam, et al. (1991) J. Immunol. **147**:1338-1343); Ravetch, J.V. and Perussia, B. (1989) J. Exp. Med. **170**:481-497; Koene, H.R. et al. (1997) Blood **90**:1109-1114 and Wu, J. et al. (1997) J. Clin. Invest. **100**:1059-1070.

Minor variations in the FcγR protein sequences (polymorphisms) have been linked to individual variation to disease susceptibility and therapeutic response, e.g., the clearance of red cells by monoclonal antibodies (Kumpel, et al. (2003) supra and Miescher, S. et al.

(2004) Blood **103(11)**:1503-1504), the onset and course of systemic lupus erythematosus (SLE) (Dijstelbloem, H.M. et al. (2000) Arthritis Rhem. **43(12)**:2793-2800); the sensitivity and therefore responsiveness to Rituximab (Mabther, Rituxan) therapy (Carton et al. (2002) supra and Weng and Levy (2003) J. Clin. Oncol. **21(21)**:3940-3947); the susceptibility to malaria (Omi, K. et al. (2002) Parasitol. Int. **51(4)**:361-366); the susceptibility to childhood immune thrombocytopenic purpura (ITP) (Carcao, M.D. et al. (2003) Br. J. Haematol. **129(1)**:135-141) and the susceptibility to advance peripheral atherosclerosis (van der Meer, I.M. et al. (2004) Throm. Haemost. **92(6)**:1273-1276). However, the relationship between FcγRIIa131 and/or FcγRIIIa 158 polymorphisms and clinical response to Cetuximab therapy has not been reported.

This invention provides methods to determine if a cancer patient expressing EGFR will be suitably treated with anti-EGFR IgG1 antibody therapy (e.g., Cetuximab). The method requires identifying the FcγRIIa 131 polymorphism that Applicant has shown to be clinically relevant to the choice of therapy to treat cancer in human patients. If a patient is H/H or H/R at position 131 of FcγRIIa, the patient is more likely to be successfully treated with anti-EGFR IgG1 antibody therapy (e.g., Cetuximab). However, Applicant has also determined that use of an anti-EGFR IgG2 antibody therapy is not likely to provide a therapeutic response such as extended survival time or a reduction in other clinical symptoms of cancer.

In one aspect, the method requires determining the presence or absence of allelic variant of the Fc γRIIa gene at positions that encode amino acid at position 131. In another aspect, the method requires determining whether the epidermal growth factor receptor (EGFR) gene is over- or under-expressed as compared to a control. In yet a further aspect, one or more of the above-noted markers is/are identified in the method of this invention.

In a further aspect, Applicant provides methods to determine if a cancer patient will be suitably treated with anti-EGFR IgG1 antibody therapy (e.g., Cetuximab). The method requires identifying the FcγRIIa 131 and FcγRIIIa 158 polymorphisms that Applicant has shown to be clinically relevant to the choice of therapy to treat cancer in human patients. If a patient is H/H or H/R at position 131 of Fc γRIIa and/or F/V at FcγRIIIa 158, the patient is more likely to be successfully treated with anti-EGFR IgG1 antibody therapy (e.g., Cetuximab). However, Applicant has also determined that use of an anti-EGFR

IgG2 antibody therapy is not likely to provide a therapeutic response such as extended survival time or a reduction in other clinical symptoms of cancer.

In one aspect, the method requires determining the identity of allelic variant of the FcγRIIa gene at amino acid position 131. In another, aspect the method requires determining the identity of allelic variant of the FcγRIIIa gene at amino acid at position 158. In yet another aspect, the method requires determining whether the epidermal growth factor receptor (EGFR) gene is over- or under-expressed as compared to a control. In yet a further aspect, one or more of the above-noted markers is/are identified in the method of this invention.

In a yet further aspect, the patients are pre-screened to determine if they express EGFR.

The invention also provides the tools to perform the methods of this invention. In one aspect, the tools include nucleic acids encompassing the polymorphic region of interest or adjacent to the polymorphic region as probes or primers and instructions for use. In another aspect, the tools detect mRNA levels of a gene of interest, e.g., EGFR. In yet further aspect, the tools include antibodies to detect protein expression levels and/or receptor expression levels of EFGR.

While the specific experimental embodiments have focused on metastatic colorectal carcinoma, the methods of this invention are not so limited. In one aspect, the cancer is treatable by blocking or inhibiting one or more members of the Epidermal Growth Factor Receptor (EGFR) pathway. Non-limiting examples of such cancers include, but are not limited to rectal cancer, colorectal cancer, metastatic colorectal cancer, colon cancer, gastric cancer, lung cancer, non-small cell lung cancer and esophageal cancers.

In one aspect, the polymorphism of interest is present in a sample. The sample can be tumor tissue. In another aspect the sample can be normal tissue isolated adjacent to the tumor. In a further aspect, the sample is any tissue of the patient, and can include peripheral blood lymphocytes.

In another aspect, the invention comprises administration of an appropriate therapy or combination therapy after identification of the polymorphism of interest.

In yet a further embodiment, the invention provides a kit for amplifying and/or for determining the molecular structure of at least a portion of the gene of interest, comprising a probe or primer capable of detecting to the gene of interest and instructions for use. In one embodiment, the probe or primer is capable of detecting to an allelic variant of the gene of interest, e.g., the FcγRIIa gene at amino acid position 131 and/or FcγRIIIa gene at amino acid position 158. In other aspect, the probe or primer is used to determine the expression level of the gene of interest, EGFR. In yet a further embodiment, the kit contains a molecule, such as an antibody, that can detect the expression product of the gene of interest, EGFR.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 graphically shows the estimated probability of survival as a function of months since start of Cetuximab therapy for FcγRIIa 131 polymorphism types. Median survival was highest for patients having H/R FcγRIIa 131 polymorphism and lowest for patients with R/R FcγRIIa 131 polymorphism. The line most distal to the axes intersection shows survival probability as measured in months of therapy for patients of genotype H/R (n=17) with median survival 12.0 (95 %CI: range of 2.7-15.4 months). The line adjacent to the intersection of the axes shows survival probability as measured in months of therapy for patients of genotype R/R (n=9) with medial survival of 2.3 months (range of 1.2 to 15.0 months). The medial line shows survival probability as measured in months of therapy for patients of genotype H/H (n=9) with medial survival 4.5 (range of 0.8 to 8.7). Log rank P value = 0.22.

Figure 2 graphically shows the estimated probability of survival as a function of months since start of Cetuximab therapy for FcγRIIa 131 and FcγRIIIa 158 polymorphisms. Median survival was highest for patients having genotype H/H or H/R FcγRIIa 131 polymorphism and lowest for patients with R/R FcγRIIa 131 polymorphism and highest for patients having genotype F/F or F/V FcγRIIIa 158 and lowest for patients having genotype V/V FcγRIIIa 158. The line distal to the intersection of the axis shows survival probability as measured in months of therapy for patients of genotype FcγRIIa 131 H/H or H/R and F/F or F/V FcγRIIIa 158 (n= 22 patient samples). The lines adjacent to the intersection shows survival probability as measured in months of therapy for patients of

genotype FcγRIIa 131 R/R and FcγRIIIa V/V 158 (n= 13 patient samples. Log rank p value is 0.004.

MODES FOR CARRYING OUT THE INVENTION

The present invention provides methods and kits for determining a subject's cancer risk and likely response to specific cancer treatment by determining the subject's genotype at the gene of interest and/or the level of transcription of a gene of interest. Other aspects of the invention are described below or will be apparent to one of skill in the art in light of the present disclosure.

Throughout this disclosure, various publications, patents and published patent specifications are referenced by an identifying citation. The disclosures of these publications, patents and published patent specifications are hereby incorporated by reference into the present disclosure to more fully describe the state of the art to which this invention pertains.

The practice of the present invention employs, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are within the skill of the art. Such techniques are explained fully in the literature for example in the following publications. *See*, e.g., Sambrook et al. MOLECULAR CLONING: A LABORATORY MANUAL, 2nd edition (1989); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (F. M. Ausubel et al. eds. (1987)); the series METHODS IN ENZYMOLOGY (Academic Press, Inc., N.Y.); PCR: A PRACTICAL APPROACH (M. MacPherson et al. IRL Press at Oxford University Press (1991)); PCR 2: A PRACTICAL APPROACH (M.J. MacPherson, B.D. Hames and G.R. Taylor eds. (1995)); ANTIBODIES, A LABORATORY MANUAL (Harlow and Lane eds. (1988)); ANIMAL CELL CULTURE (R.I. Freshney ed. (1987)); OLIGONUCLEOTIDE SYNTHESIS (M. J. Gait ed. (1984)); Mullis et al. U.S. Patent No. 4,683,195; NUCLEIC ACID HYBRIDIZATION (B. D. Hames & S. J. Higgins eds. (1984)); TRANSCRIPTION AND TRANSLATION (B. D. Hames & S. J. Higgins eds. (1984)); IMMOBILIZED CELLS AND ENZYMES (IRL Press (1986)); B. Perbal, A PRACTICAL GUIDE TO MOLECULAR CLONING (1984); GENE TRANSFER VECTORS FOR MAMMALIAN CELLS (J. H. Miller and M. P. Calos eds. (1987) Cold

Spring Harbor Laboratory); IMMUNOCHEMICAL METHODS IN CELL AND MOLECULAR BIOLOGY (Mayer and Walker, eds., Academic Press, London (1987)); HANDBOOK OF EXPERIMENTAL IMMUNOLOGY, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds. (1986)); MANIPULATING THE MOUSE EMBRYO (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1986)).

Definitions

As used herein, certain terms may have the following defined meanings. As used in the specification and claims, the singular form "a," "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a cell" includes a plurality of cells, including mixtures thereof.

As used herein, the term "comprising" is intended to mean that the compositions and methods include the recited elements, but not excluding others. "Consisting essentially of" when used to define compositions and methods, shall mean excluding other elements of any essential significance to the combination. Thus, a composition consisting essentially of the elements as defined herein would not exclude trace contaminants from the isolation and purification method and pharmaceutically acceptable carriers, such as phosphate buffered saline, preservatives, and the like. "Consisting of" shall mean excluding more than trace elements of other ingredients and substantial method steps for administering the compositions of this invention. Embodiments defined by each of these transition terms are within the scope of this invention.

All numerical designations, e.g., pH, temperature, time, concentration, and molecular weight, including ranges, are approximations which are varied (+) or (-) by increments of 0.1. It is to be understood, although not always explicitly stated that all numerical designations are preceded by the term "about". It also is to be understood, although not always explicitly stated, that the reagents described herein are merely exemplary and that equivalents of such are known in the art.

The term "antigen" is well understood in the art and includes substances which are immunogenic. The EGFR is an example of an antigen. The term as used herein also includes substances which induce immunological unresponsiveness or anergy.

"EGFR" , also called "HER-1" is a transmembrane glycoprotein that binds specific ligands, EGF and transforming growth factor alpha (α) to the extracellular domain leading to the dimerization of the receptor with another EGFR (homodimerization) or another member of the EGFR family (heterodimerization). Upon dimermization, phoshphorylation of the intracellular tyrosine kinases of the receptor, initiating a cascade of intracellular signaling that regulates cellular processes such as proliferation, migration, adhesion, differentiation and survival. Carpenter, G. et al. (1990) J. Biol. Chem. **265**:7709-7712; Real, F.X. et al. (1986) Cancer Res. **46**:4726-4731; and Ciardiello, F. and Tortora, G. (2001) Clin. Cancer Res. **7**:2958-2970.

A "native" or "natural" or "wild-type" antigen is a polypeptide, protein or a fragment which contains an epitope and which has been isolated from a natural biological source. It also can specifically bind to an antigen receptor.

As used herein, an "antibody" includes whole antibodies and any antigen binding fragment or a single chain thereof. Thus the term "antibody" includes any protein or peptide containing molecule that comprises at least a portion of an immunoglobulin molecule. Examples of such include, but are not limited to a complementarity determining region (CDR) of a heavy or light chain or a ligand binding portion thereof, a heavy chain or light chain variable region, a heavy chain or light chain constant region, a framework (FR) region, or any portion thereof, or at least one portion of a binding protein, any of which can be incorporated into an antibody of the present invention.

The antibodies can be polyclonal or monoclonal and can be isolated from any suitable biological source, e.g., murine, rat, sheep and canine. Additional sources are identified infra.

In one aspect, the "biological activity" means the ability of the antibody to selectively bind its epitope protein or fragment thereof as measured by ELISA or other suitable methods. Biologically equivalent antibodies, include but are not limited to those antibodies, peptides, antibody fragments, antibody variant, antibody derivative and antibody mimetics that bind to the same epitope as the reference antibody.

The term "antibody" is further intended to encompass digestion fragments, specified portions, derivatives and variants thereof, including antibody mimetics or comprising

portions of antibodies that mimic the structure and/or function of an antibody or specified fragment or portion thereof, including single chain antibodies and fragments thereof. Examples of binding fragments encompassed within the term "antigen binding portion" of an antibody include a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH, domains; a $F(ab')^2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; a Fd fragment consisting of the VH and CH, domains; a Fv fragment consisting of the VL and VH domains of a single arm of an antibody, a dAb fragment (Ward et al. (1989) Nature **341**:544-546), which consists of a VH domain; and an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, VL and VH, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VL and VH regions pair to form monovalent molecules (known as single chain Fv (scFv)). Bird et al. (1988) Science **242**:423-426 and Huston et al. (1988) Proc. Natl. Acad Sci. USA **85**:5879-5883. Single chain antibodies are also intended to be encompassed within the term "fragment of an antibody." Any of the above-noted antibody fragments are obtained using conventional techniques known to those of skill in the art, and the fragments are screened for binding specificity and neutralization activity in the same manner as are intact antibodies.

The term "epitope" means a protein determinant capable of specific binding to an antibody. Epitopes usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics. Conformational and nonconformational epitopes are distinguished in that the binding to the former but not the latter is lost in the presence of denaturing solvents.

The term "antibody variant" is intended to include antibodies produced in a species other than a mouse. It also includes antibodies containing post-translational modifications to the linear polypeptide sequence of the antibody or fragment. It further encompasses fully human antibodies.

The term "antibody derivative" is intended to encompass molecules that bind an epitope as defined above and which are modifications or derivatives of a native monoclonal antibody of this invention. Derivatives include, but are not limited to, for example, bispecific,

multispecific, heterospecific, trispecific, tetraspecific, multispecific antibodies, diabodies, chimeric, recombinant and humanized.

The term "bispecific molecule" is intended to include any agent, e.g., a protein, peptide, or protein or peptide complex, which has two different binding specificities. The term "multispecific molecule" or "heterospecific molecule" is intended to include any agent, e.g. a protein, peptide, or protein or peptide complex, which has more than two different binding specificities.

The term "heteroantibodies" refers to two or more antibodies, antibody binding fragments (e.g., Fab), derivatives thereof, or antigen binding regions linked together, at least two of which have different specificities.

The term "human antibody" as used herein, is intended to include antibodies having variable and constant regions derived from human germline immunoglobulin sequences. The human antibodies of the invention may include amino acid residues not encoded by human germline immunoglobulin sequences (e.g., mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*). However, the term "human antibody" as used herein, is not intended to include antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences. Thus, as used herein, the term "human antibody" refers to an antibody in which substantially every part of the protein (e.g., CDR, framework, C_L, C_H domains (e.g., C_{H1}, C_{H2}, C_{H3}), hinge, (V_L, V_H)) is substantially non-immunogenic in humans, with only minor sequence changes or variations. Similarly, antibodies designated primate (monkey, baboon, chimpanzee, etc.), rodent (mouse, rat, rabbit, guinea pig, hamster, and the like) and other mammals designate such species, sub-genus, genus, sub-family, family specific antibodies. Further, chimeric antibodies include any combination of the above. Such changes or variations optionally and preferably retain or reduce the immunogenicity in humans or other species relative to non-modified antibodies. Thus, a human antibody is distinct from a chimeric or humanized antibody. It is pointed out that a human antibody can be produced by a non-human animal or prokaryotic or eukaryotic cell that is capable of expressing functionally rearranged human immunoglobulin (e.g., heavy chain and/or light chain) genes. Further, when a human antibody is a single chain antibody, it can comprise a linker peptide that is not found in

native human antibodies. For example, an Fv can comprise a linker peptide, such as two to about eight glycine or other amino acid residues, which connects the variable region of the heavy chain and the variable region of the light chain. Such linker peptides are considered to be of human origin.

As used herein, a human antibody is "derived from" a particular germline sequence if the antibody is obtained from a system using human immunoglobulin sequences, e.g., by immunizing a transgenic mouse carrying human immunoglobulin genes or by screening a human immunoglobulin gene library. A human antibody that is "derived from" a human germline immunoglobulin sequence can be identified as such by comparing the amino acid sequence of the human antibody to the amino acid sequence of human germline immunoglobulins. A selected human antibody typically is at least 90% identical in amino acids sequence to an amino acid sequence encoded by a human germline immunoglobulin gene and contains amino acid residues that identify the human antibody as being human when compared to the germline immunoglobulin amino acid sequences of other species (e.g., murine germline sequences). In certain cases, a human antibody may be at least 95%, or even at least 96%, 97%, 98%, or 99% identical in amino acid sequence to the amino acid sequence encoded by the germline immunoglobulin gene. Typically, a human antibody derived from a particular human germline sequence will display no more than 10 amino acid differences from the amino acid sequence encoded by the human germline immunoglobulin gene. In certain cases, the human antibody may display no more than 5, or even no more than 4, 3, 2, or 1 amino acid difference from the amino acid sequence encoded by the germline immunoglobulin gene.

The terms "monoclonal antibody" or "monoclonal antibody composition" as used herein refer to a preparation of antibody molecules of single molecular composition. A monoclonal antibody composition displays a single binding specificity and affinity for a particular epitope.

A "human monoclonal antibody" refers to antibodies displaying a single binding specificity which have variable and constant regions derived from human germline immunoglobulin sequences.

The term "recombinant human antibody", as used herein, includes all human antibodies that are prepared, expressed, created or isolated by recombinant means, such as antibodies isolated from an animal (e.g., a mouse) that is transgenic or transchromosomal for human immunoglobulin genes or a hybridoma prepared therefrom, antibodies isolated from a host cell transformed to express the antibody, e.g., from a transfectoma, antibodies isolated from a recombinant, combinatorial human antibody library, and antibodies prepared, expressed, created or isolated by any other means that involve splicing of human immunoglobulin gene sequences to other DNA sequences. Such recombinant human antibodies have variable and constant regions derived from human germline immunoglobulin sequences. In certain embodiments, however, such recombinant human antibodies can be subjected to *in vitro* mutagenesis (or, when an animal transgenic for human Ig sequences is used, *in vivo* somatic mutagenesis) and thus the amino acid sequences of the VH and VL regions of the recombinant antibodies are sequences that, while derived from and related to human germline VH and VL sequences, may not naturally exist within the human antibody germline repertoire *in vivo*.

As used herein, "isotype" refers to the antibody class (e.g., IgM or IgG1) that is encoded by heavy chain constant region genes.

The term "allele", which is used interchangeably herein with "allelic variant" refers to alternative forms of a gene or portions thereof. Alleles occupy the same locus or position on homologous chromosomes. When a subject has two identical alleles of a gene, the subject is said to be homozygous for the gene or allele. When a subject has two different alleles of a gene, the subject is said to be heterozygous for the gene. Alleles of a specific gene can differ from each other in a single nucleotide, or several nucleotides, and can include substitutions, deletions and insertions of nucleotides. An allele of a gene can also be a form of a gene containing a mutation.

The terms "protein", "polypeptide" and "peptide" are used interchangeably herein when referring to a gene product.

The term "recombinant protein" refers to a polypeptide which is produced by recombinant DNA techniques, wherein generally, DNA encoding the polypeptide is inserted into a

suitable expression vector which is in turn used to transform a host cell to produce the heterologous protein.

As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of preferred vector is an episome, i.e., a nucleic acid capable of extra-chromosomal replication. Preferred vectors are those capable of autonomous replication and/or expression of nucleic acids to which they are linked. Vectors capable of directing the expression of genes to which they are operatively linked are referred to herein as "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of "plasmids" which refer generally to circular double stranded DNA loops which, in their vector form are not bound to the chromosome. In the present specification, "plasmid" and "vector" are used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which become known in the art subsequently hereto.

The term "wild-type allele" refers to an allele of a gene which, when present in two copies in a subject results in a wild-type phenotype. There can be several different wild-type alleles of a specific gene, since certain nucleotide changes in a gene may not affect the phenotype of a subject having two copies of the gene with the nucleotide changes.

The term "allelic variant of a polymorphic region of the gene of interest" refers to a region of the gene of interest having one of a plurality of nucleotide sequences found in that region of the gene in other individuals.

"Cells," "host cells" or "recombinant host cells" are terms used interchangeably herein. It is understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

The expression "amplification of polynucleotides" includes methods such as PCR, ligation amplification (or ligase chain reaction, LCR) and amplification methods. These methods are known and widely practiced in the art. See, e.g., U.S. Pat. Nos. 4,683,195 and

4,683,202 and Innis et al., 1990 (for PCR); and Wu, D.Y. et al. (1989) Genomics 4:560-569 (for LCR). In general, the PCR procedure describes a method of gene amplification which is comprised of (i) sequence-specific hybridization of primers to specific genes within a DNA sample (or library), (ii) subsequent amplification involving multiple rounds of annealing, elongation, and denaturation using a DNA polymerase, and (iii) screening the PCR products for a band of the correct size. The primers used are oligonucleotides of sufficient length and appropriate sequence to provide initiation of polymerization, i.e. each primer is specifically designed to be complementary to each strand of the genomic locus to be amplified.

Reagents and hardware for conducting PCR are commercially available. Primers useful to amplify sequences from a particular gene region are preferably complementary to, and hybridize specifically to sequences in the target region or in its flanking regions. Nucleic acid sequences generated by amplification may be sequenced directly. Alternatively the amplified sequence(s) may be cloned prior to sequence analysis. A method for the direct cloning and sequence analysis of enzymatically amplified genomic segments is known in the art.

The term "encode" as it is applied to polynucleotides refers to a polynucleotide which is said to "encode" a polypeptide if, in its native state or when manipulated by methods well known to those skilled in the art, it can be transcribed and/or translated to produce the mRNA for the polypeptide and/or a fragment thereof. The antisense strand is the complement of such a nucleic acid, and the encoding sequence can be deduced therefrom.

The term "genotype" refers to the specific allelic composition of an entire cell or a certain gene, whereas the term "phenotype' refers to the detectable outward manifestations of a specific genotype.

As used herein, the term "gene" or "recombinant gene" refers to a nucleic acid molecule comprising an open reading frame and including at least one exon and (optionally) an intron sequence. The term "intron" refers to a DNA sequence present in a given gene which is spliced out during mRNA maturation.

"Homology" or "identity" or "similarity" refers to sequence similarity between two peptides or between two nucleic acid molecules. Homology can be determined by

comparing a position in each sequence which may be aligned for purposes of comparison. When a position in the compared sequence is occupied by the same base or amino acid, then the molecules are homologous at that position. A degree of homology between sequences is a function of the number of matching or homologous positions shared by the sequences. An "unrelated" or "non-homologous" sequence shares less than 40% identity, though preferably less than 25% identity, with one of the sequences of the present invention.

The term "a homolog of a nucleic acid" refers to a nucleic acid having a nucleotide sequence having a certain degree of homology with the nucleotide sequence of the nucleic acid or complement thereof. A homolog of a double stranded nucleic acid is intended to include nucleic acids having a nucleotide sequence which has a certain degree of homology with or with the complement thereof. In one aspect, homologs of nucleic acids are capable of hybridizing to the nucleic acid or complement thereof.

The term "interact" as used herein is meant to include detectable interactions between molecules, such as can be detected using, for example, a hybridization assay. The term interact is also meant to include "binding" interactions between molecules. Interactions may be, for example, protein-protein, protein-nucleic acid, protein-small molecule or small molecule-nucleic acid in nature.

The term "isolated" as used herein with respect to nucleic acids, such as DNA or RNA, refers to molecules separated from other DNAs or RNAs, respectively, that are present in the natural source of the macromolecule. The term isolated as used herein also refers to a nucleic acid or peptide that is substantially free of cellular material, viral material, or culture medium when produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized. Moreover, an "isolated nucleic acid" is meant to include nucleic acid fragments which are not naturally occurring as fragments and would not be found in the natural state. The term "isolated" is also used herein to refer to polypeptides which are isolated from other cellular proteins and is meant to encompass both purified and recombinant polypeptides.

The term "mismatches" refers to hybridized nucleic acid duplexes which are not 100% homologous. The lack of total homology may be due to deletions, insertions, inversions, substitutions or frameshift mutations.

As used herein, the term "nucleic acid" refers to polynucleotides such as deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA). The term should also be understood to include, as equivalents, derivatives, variants and analogs of either RNA or DNA made from nucleotide analogs, and, as applicable to the embodiment being described, single (sense or antisense) and double-stranded polynucleotides. Deoxyribonucleotides include deoxyadenosine, deoxycytidine, deoxyguanosine, and deoxythymidine. For purposes of clarity, when referring herein to a nucleotide of a nucleic acid, which can be DNA or an RNA, the terms "adenosine", "cytidine", "guanosine", and thymidine" are used. It is understood that if the nucleic acid is RNA, a nucleotide having a uracil base is uridine.

The terms "oligonucleotide" or "polynucleotide", or "portion," or "segment" thereof refer to a stretch of polynucleotide residues which is long enough to use in PCR or various hybridization procedures to identify or amplify identical or related parts of mRNA or DNA molecules. The polynucleotide compositions of this invention include RNA, cDNA, genomic DNA, synthetic forms, and mixed polymers, both sense and antisense strands, and may be chemically or biochemically modified or may contain non-natural or derivatized nucleotide bases, as will be readily appreciated by those skilled in the art. Such modifications include, for example, labels, methylation, substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, *etc.*), charged linkages (e.g., phosphorothioates, phosphorodithioates, *etc.*), pendent moieties (e.g., polypeptides), intercalators (e.g., acridine, psoralen, *etc.*), chelators, alkylators, and modified linkages (e.g., alpha anomeric nucleic acids, *etc.*). Also included are synthetic molecules that mimic polynucleotides in their ability to bind to a designated sequence via hydrogen bonding and other chemical interactions. Such molecules are known in the art and include, for example, those in which peptide linkages substitute for phosphate linkages in the backbone of the molecule.

The term "polymorphism" refers to the coexistence of more than one form of a gene or portion thereof. A portion of a gene of which there are at least two different forms, i.e., two different nucleotide sequences, is referred to as a "polymorphic region of a gene". A polymorphic region can be a single nucleotide, the identity of which differs in different alleles.

A "polymorphic gene" refers to a gene having at least one polymorphic region.

The term "treating" as used herein is intended to encompass curing as well as ameliorating at least one symptom of the condition or disease. For example, in the case of cancer, treatment includes a reduction in cachexia, increase in survival time, elongation in time to tumor progression, reduction in tumor mass, reduction in tumor burden and/or a prolongation in time to tumor metastasis, each as measured by standards set by the National Cancer Institute and the U.S. Food and Drug Administration for the approval of new drugs. See Johnson et al. (2003) J. Clin. Oncol. **21(7)**:1404-1411.

A "complete response" (CR) to a therapy defines patients with evaluable but non-measurable disease, whose tumor and all evidence of disease had disappeared.

A "partial response" (PR) to a therapy defines patients with anything less than complete response were simply categorized as demonstrating partial response.

"Stable disease" (SD) indicates that the patient is stable.

"Non-response" (NR) to a therapy defines patients whose tumor or evidence of disease has remained constant or has progressed.

This invention provides a method for selecting a therapeutic regimen or determining if a certain therapeutic regimen is more likely to treat a cancer or is the appropriate chemotherapy for that patient than other available chemotherapies. In general, a therapy is considered to "treat" cancer if it provides one or more of the following treatment outcomes: reduce or delay recurrence of the cancer after the initial therapy; increase median survival time or decrease metastases. The method is particularly suited to determining which patients will be responsive or experience a positive treatment outcome to an anti-EGFR IgG1 antibody therapy, such as Certuximab. These methods are useful to

diagnose or predict individual responsiveness to any cancer that has been treatable with these therapies, for example, highly aggressive cancers such as colorectal cancer.

In one embodiment, the chemotherapeutic regimen further comprises radiation therapy or other suitable therapy.

The method comprises screening for a genomic polymorphism or genotype of the FcγRIIa or FcγRIIIa gene that has been correlated by Applicant to be clinically relevant. In one aspect, the method also requires isolating a sample containing the genetic material to be tested; however, it is conceivable that one of skill in the art will be able to analyze and identify genetic polymorphisms in situ at some point in the future. Accordingly, the inventions of this application are not to be limited to requiring isolation of the genetic material prior to analysis.

The genomic polymorphisms that have been correlated to susceptibility to IgG1 antibodies (e.g., anti-EGFR therapies such as Cetuximab) or mimetics having the same mechanism of action. In one aspect the method also identifies patients that are not suitably treated with anti-IgG2-EGFR antibodies or mimetics or equivalents.

This invention provides methods to determine if a cancer patient will be suitably treated with anti-EGFR IgG1 antibody therapy (e.g., Cetuximab). The method requires identifying the amino acid at FcγRIIa 131 that Applicant has shown to be clinically relevant to the choice of therapy to treat cancer in human patients. If a patient is H/H or H/R at amino acid position 131 of FcγRIIa, the patient is more likely to be successfully treated with anti-EGFR IgG1 antibody therapy (e.g., Cetuximab). However, Applicant has also determined that use of an anti-EGFR IgG2 antibody therapy is not likely to provide a therapeutic response such as extended survival time or a reduction in other clinical symptoms of cancer.

In another aspect, the method requires determining the identity of one or more of the following polymorphisims: FcγRIIIa 158 or FcγRIIa 131, or yet further the expression level of the EGFR gene. In one aspect, the identification or identity of FcγRIIa 131 position (e.g., FcγRIIa 131 H/R polymorphism). In a further embodiment, the FcγRIIIa 158 position (e.g., FcγRIIIa V/F polymorphism) is tested. In yet a further aspect, the presence, absence or level of expression of the EGFR gene is further obtained.

Applicant has determined that patients with FcγRIIa 131 H/H or H/R polymorphisms show better time to progression (p=0.037,log-rank test) and overall survival as compared to patients with R/R polymorphisms (p=0.22, log-rank test) after treatment with Certuximab. Applicant also determined that a trend exists in significance of tumor response to therapy when patients with R/R polymorphisms were compared to patients with H/H or H/R polymorphism at this position (p=0.08, fisher exact test). Although an initial study with a very small patient sample did not show a correlation between FcγRIIIa 158 and clinical outcome, after enlargement of the patient pool a clinical correlation was found.

Prior investigators reported a correlation between the 131 and 158 two polymorphisms and responsiveness to Rituximab therapy. Weng and Levy (2003) supra, reporting on the reported findings of Cartron et al. (2002) Blood **99**:754-758 and reviews by Yan and Beckman (2005) BioTechniques **39**:565-568 and Chung and Saltz (2005) The Oncologist **10**:701-709.

The method is useful to select treatments for a cancer or neoplasm selected from the group consisting of esophageal cancer, gastric cancer, colon cancer, rectal cancer, colorectal cancer, metastatic colorectal cancer, lung cancer and non-small cell lung cancer (NSCLC). In yet a further aspect, the cancers are present in patients with low or no expression of the EGFR gene. See Chung and Saltz (2005) supra. In a futher aspect, the patient sample contains cells expression EGFR. In one aspect, the cancer is colorectal colon cancer and in yet a further aspect, it is metastatic colorectal colon cancer.

The method can be used to predict responsiveness to IgG1-antibody (e.g., Certuximab or similar therapy) as a first line treatment, or alternatively for patients that have not been treated with on or more prior therapies, e.g., patients who have failed prior CPT-11 (Irinotecan), 5-Fluorouracil (5-FU) with or without leucovorin ("LV") and oxaliplatin therapy. In yet a further aspect, patients have failed one or more prior therapy selected from the groups consisting of CPT-11/ 5-FU, LV and oxaliplatin therapy.

Diagnostic Methods

The invention further features predictive medicines, which are based, at least in part, on determination of the identity of the polymorphic region or expression level (or both in combination) of the FcγRII 131 polymorphism and/or the FcγRIII 158 polymorphism.

For example, information obtained using the diagnostic assays described herein is useful for determining if a subject will respond to cancer treatment of a given type. Based on the prognostic information, a doctor can recommend a regimen (e.g. diet or exercise) or therapeutic protocol, useful for treating cancer in the individual.

In addition, knowledge of the identity of a particular allele in an individual (the gene profile) allows customization of therapy for a particular disease to the individual's genetic profile, the goal of "pharmacogenomics". For example, an individual's genetic profile can enable a doctor: 1) to more effectively prescribe a drug that will address the molecular basis of the disease or condition; 2) to better determine the appropriate dosage of a particular drug and 3) to identify novel targets for drug development. Expression patterns of individual patients can then be compared to the expression profile of the disease to determine the appropriate drug and dose to administer to the patient.

The ability to target populations expected to show the highest clinical benefit, based on the normal or disease genetic profile, can enable: 1) the repositioning of marketed drugs with disappointing market results; 2) the rescue of drug candidates whose clinical development has been discontinued as a result of safety or efficacy limitations, which are patient subgroup-specific; and 3) an accelerated and less costly development for drug candidates and more optimal drug labeling.

Detection of point mutations can be accomplished by molecular cloning of the specified allele and subsequent sequencing of that allele using techniques known in the art. Alternatively, the gene sequences can be amplified directly from a genomic DNA preparation from the tumor tissue using PCR, and the sequence composition is determined from the amplified product. As described more fully below, numerous methods are available for analyzing a subject's DNA for mutations at a given genetic locus such as the gene of interest.

A detection method is allele specific hybridization using probes overlapping the polymorphic site and having about 5, or alternatively 10, or alternatively 20, or alternatively 25, or alternatively 30 nucleotides around the polymorphic region. In another embodiment of the invention, several probes capable of hybridizing specifically to the allelic variant are attached to a solid phase support, e.g., a "chip". Oligonucleotides can be bound to a solid support by a variety of processes, including lithography. For example a chip can hold up to 250,000 oligonucleotides (GeneChip, Affymetrix). Mutation detection analysis using these chips comprising oligonucleotides, also termed "DNA probe arrays" is described e.g., in Cronin et al. (1996) Human Mutation **7**:244.

In other detection methods, it is necessary to first amplify at least a portion of the gene of interest prior to identifying the allelic variant. Amplification can be performed, e.g., by PCR and/or LCR, according to methods known in the art. In one embodiment, genomic DNA of a cell is exposed to two PCR primers and amplification for a number of cycles sufficient to produce the required amount of amplified DNA.

Alternative amplification methods include: self sustained sequence replication (Guatelli, J. C. et al. (1990) Proc. Natl. Acad. Sci. USA **87**:1874-1878), transcriptional amplification system (Kwoh, D. Y. et al., (1989) Proc. Natl. Acad. Sci. USA **86**:1173-1177), Q-Beta Replicase (Lizardi, P. M. et al. (1988) Bio/Technology **6**:1197), or any other nucleic acid amplification method, followed by the detection of the amplified molecules using techniques known to those of skill in the art. These detection schemes are useful for the detection of nucleic acid molecules if such molecules are present in very low numbers.

In one embodiment, any of a variety of sequencing reactions known in the art can be used to directly sequence at least a portion of the gene of interest and detect allelic variants, e.g., mutations, by comparing the sequence of the sample sequence with the corresponding wild-type (control) sequence. Exemplary sequencing reactions include those based on techniques developed by Maxam and Gilbert ((1997) Proc. Natl Acad Sci, USA **74**:560) or Sanger (Sanger et al. (1977) Proc. Nat. Acad. Sci, **74**:5463). It is also contemplated that any of a variety of automated sequencing procedures can be utilized when performing the subject assays (Biotechniques (1995) **19**:448), including sequencing by mass spectrometry (see, for example, U.S. Patent No. 5,547,835 and International Patent Application Publication Number WO94/16101, entitled DNA Sequencing by Mass Spectrometry by H.

Koster; U.S. Patent No. 5,547,835 and international patent application Publication Number WO 94/21822 entitled "DNA Sequencing by Mass Spectrometry Via Exonuclease Degradation" by H. Koster; U.S. Patent No. 5,605,798 and International Patent Application No. PCT/US96/03651 entitled DNA Diagnostics Based on Mass Spectrometry by H. Koster; Cohen et al. (1996) Adv. Chromat. **36**:127-162; and Griffin et al. (1993) Appl Biochem Bio. **38**:147-159). It will be evident to one skilled in the art that, for certain embodiments, the occurrence of only one, two or three of the nucleic acid bases need be determined in the sequencing reaction. For instance, A-track or the like, e.g., where only one nucleotide is detected, can be carried out.

Yet other sequencing methods are disclosed, e.g., in U.S. Patent No. 5,580,732 entitled "Method Of DNA Sequencing Employing A Mixed DNA-Polymer Chain Probe" and U.S. Patent No. 5,571,676 entitled "Method For Mismatch-Directed *In Vitro* DNA Sequencing."

In some cases, the presence of the specific allele in DNA from a subject can be shown by restriction enzyme analysis. For example, the specific nucleotide polymorphism can result in a nucleotide sequence comprising a restriction site which is absent from the nucleotide sequence of another allelic variant.

In a further embodiment, protection from cleavage agents (such as a nuclease, hydroxylamine or osmium tetroxide and with piperidine) can be used to detect mismatched bases in RNA/RNA DNA/DNA, or RNA/DNA heteroduplexes (see, e.g., Myers et al. (1985) Science **230**:1242). In general, the technique of "mismatch cleavage" starts by providing heteroduplexes formed by hybridizing a control nucleic acid, which is optionally labeled, e.g., RNA or DNA, comprising a nucleotide sequence of the allelic variant of the gene of interest with a sample nucleic acid, e.g., RNA or DNA, obtained from a tissue sample. The double-stranded duplexes are treated with an agent which cleaves single-stranded regions of the duplex such as duplexes formed based on basepair mismatches between the control and sample strands. For instance, RNA/DNA duplexes can be treated with RNase and DNA/DNA hybrids treated with S1nuclease to enzymatically digest the mismatched regions. In other embodiments, either DNA/DNA or RNA/DNA duplexes can be treated with hydroxylamine or osmium tetroxide and with piperidine in order to digest mismatched regions. After digestion of the mismatched

regions, the resulting material is then separated by size on denaturing polyacrylamide gels to determine whether the control and sample nucleic acids have an identical nucleotide sequence or in which nucleotides they are different. See, for example, U.S. Patent No. 6,455,249, Cotton et al. (1988) Proc. Natl. Acad. Sci. USA **85**:4397; Saleeba et al. (1992) Methods Enzy. **217**:286-295. In another embodiment, the control or sample nucleic acid is labeled for detection.

In other embodiments, alterations in electrophoretic mobility is used to identify the particular allelic variant. For example, single strand conformation polymorphism (SSCP) may be used to detect differences in electrophoretic mobility between mutant and wild type nucleic acids (Orita et al. (1989) Proc Natl. Acad. Sci USA **86**:2766; Cotton (1993) Mutat. Res. **285**:125-144 and Hayashi (1992) Genet Anal Tech Appl **9**:73-79). Single-stranded DNA fragments of sample and control nucleic acids are denatured and allowed to renature. The secondary structure of single-stranded nucleic acids varies according to sequence, the resulting alteration in electrophoretic mobility enables the detection of even a single base change. The DNA fragments may be labeled or detected with labeled probes. The sensitivity of the assay may be enhanced by using RNA (rather than DNA), in which the secondary structure is more sensitive to a change in sequence. In another preferred embodiment, the subject method utilizes heteroduplex analysis to separate double stranded heteroduplex molecules on the basis of changes in electrophoretic mobility (Keen et al. (1991) Trends Genet. **7**:5).

In yet another embodiment, the identity of the allelic variant is obtained by analyzing the movement of a nucleic acid comprising the polymorphic region in polyacrylamide gels containing a gradient of denaturant, which is assayed using denaturing gradient gel electrophoresis (DGGE) (Myers et al. (1985) Nature **313**:495). When DGGE is used as the method of analysis, DNA will be modified to insure that it does not completely denature, for example by adding a GC clamp of approximately 40 bp of high-melting GC-rich DNA by PCR. In a further embodiment, a temperature gradient is used in place of a denaturing agent gradient to identify differences in the mobility of control and sample DNA (Rosenbaum and Reissner (1987) Biophys Chem **265**:1275).

Examples of techniques for detecting differences of at least one nucleotide between 2 nucleic acids include, but are not limited to, selective oligonucleotide hybridization,

selective amplification, or selective primer extension. For example, oligonucleotide probes may be prepared in which the known polymorphic nucleotide is placed centrally (allele-specific probes) and then hybridized to target DNA under conditions which permit hybridization only if a perfect match is found (Saiki et al. (1986) Nature **324**:163); Saiki et al. (1989) Proc. Natl Acad. Sci USA **86**:6230 and Wallace et al. (1979) Nucl. Acids Res. **6**:3543). Such allele specific oligonucleotide hybridization techniques may be used for the detection of the nucleotide changes in the polylmorphic region of the gene of interest. For example, oligonucleotides having the nucleotide sequence of the specific allelic variant are attached to a hybridizing membrane and this membrane is then hybridized with labeled sample nucleic acid. Analysis of the hybridization signal will then reveal the identity of the nucleotides of the sample nucleic acid.

Alternatively, allele specific amplification technology which depends on selective PCR amplification may be used in conjunction with the instant invention. Oligonucleotides used as primers for specific amplification may carry the allelic variant of interest in the center of the molecule (so that amplification depends on differential hybridization) (Gibbs et al. (1989) Nucleic Acids Res. **17**:2437-2448) or at the extreme 3' end of one primer where, under appropriate conditions, mismatch can prevent, or reduce polymerase extension (Prossner (1993) Tibtech **11**:238 and Newton et al. (1989) Nucl. Acids Res. **17**:2503). This technique is also termed "PROBE" for Probe Oligo Base Extension. In addition it may be desirable to introduce a novel restriction site in the region of the mutation to create cleavage-based detection (Gasparini et al. (1992) Mol. Cell Probes **6**:1).

In another embodiment, identification of the allelic variant is carried out using an oligonucleotide ligation assay (OLA), as described, e.g., in U.S. Patent No. 4,998,617 and in Landegren, U. et al. Science **241**:1077-1080 (1988). The OLA protocol uses two oligonucleotides which are designed to be capable of hybridizing to abutting sequences of a single strand of a target. One of the oligonucleotides is linked to a separation marker, e.g., biotinylated, and the other is detectably labeled. If the precise complementary sequence is found in a target molecule, the oligonucleotides will hybridize such that their termini abut, and create a ligation substrate. Ligation then permits the labeled oligonucleotide to be recovered using avidin, or another biotin ligand. Nickerson, D. A. et al. have described a nucleic acid detection assay that combines attributes of PCR and OLA

(Nickerson, D. A. et al. (1990) Proc. Natl. Acad. Sci. (U.S.A.) **87**:8923-8927). In this method, PCR is used to achieve the exponential amplification of target DNA, which is then detected using OLA.

Several techniques based on this OLA method have been developed and can be used to detect the specific allelic variant of the polymorphic region of the gene of interest. For example, U.S. Patent No. 5,593,826 discloses an OLA using an oligonucleotide having 3'-amino group and a 5'-phosphorylated oligonucleotide to form a conjugate having a phosphoramidate linkage. In another variation of OLA described in Tobe et al. (1996)Nucleic Acids Res. **24**: 3728), OLA combined with PCR permits typing of two alleles in a single microtiter well. By marking each of the allele-specific primers with a unique hapten, i.e. digoxigenin and fluorescein, each OLA reaction can be detected by using hapten specific antibodies that are labeled with different enzyme reporters, alkaline phosphatase or horseradish peroxidase. This system permits the detection of the two alleles using a high throughput format that leads to the production of two different colors.

The invention further provides methods for detecting the single nucleotide polymorphism in the gene of interest. Because single nucleotide polymorphisms constitute sites of variation flanked by regions of invariant sequence, their analysis requires no more than the determination of the identity of the single nucleotide present at the site of variation and it is unnecessary to determine a complete gene sequence for each patient. Several methods have been developed to facilitate the analysis of such single nucleotide polymorphisms.

In one embodiment, the single base polymorphism can be detected by using a specialized exonuclease-resistant nucleotide, as disclosed, e.g., in Mundy, C. R. (U.S. Patent No. 4,656,127). According to the method, a primer complementary to the allelic sequence immediately 3' to the polymorphic site is permitted to hybridize to a target molecule obtained from a particular animal or human. If the polymorphic site on the target molecule contains a nucleotide that is complementary to the particular exonuclease-resistant nucleotide derivative present, then that derivative will be incorporated onto the end of the hybridized primer. Such incorporation renders the primer resistant to exonuclease, and thereby permits its detection. Since the identity of the exonuclease-resistant derivative of the sample is known, a finding that the primer has become resistant to exonucleases reveals that the nucleotide present in the polymorphic site of the target

molecule was complementary to that of the nucleotide derivative used in the reaction. This method has the advantage that it does not require the determination of large amounts of extraneous sequence data.

In another embodiment of the invention, a solution-based method is used for determining the identity of the nucleotide of the polymorphic site. Cohen, D. et al. (French Patent 2,650,840; PCT Appln. No. WO91/02087). As in the Mundy method of U.S. Patent No. 4,656,127, a primer is employed that is complementary to allelic sequences immediately 3' to a polymorphic site. The method determines the identity of the nucleotide of that site using labeled dideoxynucleotide derivatives, which, if complementary to the nucleotide of the polymorphic site will become incorporated onto the terminus of the primer.

An alternative method, known as Genetic Bit Analysis or GBA™ is described by Goelet, P. et al. (PCT Appln. No. 92/15712). This method uses mixtures of labeled terminators and a primer that is complementary to the sequence 3' to a polymorphic site. The labeled terminator that is incorporated is thus determined by, and complementary to, the nucleotide present in the polymorphic site of the target molecule being evaluated. In contrast to the method of Cohen et al. (French Patent 2,650,840; PCT Appln. No. WO91/02087) the method of Goelet, P. et al. supra, is preferably a heterogeneous phase assay, in which the primer or the target molecule is immobilized to a solid phase.

Recently, several primer-guided nucleotide incorporation procedures for assaying polymorphic sites in DNA have been described (Komher, J. S. et al. (1989) Nucl. Acids. Res. **17**:7779-7784; Sokolov, B. P. (1990) Nucl. Acids Res. **18**:3671; Syvanen, A.-C., et al. (1990) Genomics **8**:684-692; Kuppuswamy, M. N. et al. (1991) Proc. Natl. Acad. Sci. (U.S.A.) **88**:1143-1147; Prezant, T. R. et al. (1992) Hum. Mutat. **1**:159-164; Ugozzoli, L. et al. (1992) GATA **9**:107-112; Nyren, P. et al. (1993) Anal. Biochem. **208**:171-175). These methods differ from GBA™ in that they all rely on the incorporation of labeled deoxynucleotides to discriminate between bases at a polymorphic site. In such a format, since the signal is proportional to the number of deoxynucleotides incorporated, polymorphisms that occur in runs of the same nucleotide can result in signals that are proportional to the length of the run (Syvanen, A.-C., et al. (1993) Amer. J. Hum. Genet. **52**:46-59).

If the polymorphic region is located in the coding region of the gene of interest, yet other methods than those described above can be used for determining the identity of the allelic variant. For example, identification of the allelic variant, which encodes a mutated signal peptide, can be performed by using an antibody specifically recognizing the mutant protein in, e.g., immunohistochemistry or immunoprecipitation. Antibodies to the wild-type or signal peptide mutated forms of the signal peptide proteins can be prepared according to methods known in the art.

Antibodies directed against wild type or mutant peptides encoded by the allelic variants of the gene of interest may also be used in disease diagnostics and prognostics. Such diagnostic methods, may be used to detect abnormalities in the level of expression of the peptide, or abnormalities in the structure and/or tissue, cellular, or subcellular location of the peptide. Protein from the tissue or cell type to be analyzed may easily be detected or isolated using techniques which are well known to one of skill in the art, including but not limited to Western blot analysis. For a detailed explanation of methods for carrying out Western blot analysis, see Sambrook et al., (1989) supra, at Chapter 18. The protein detection and isolation methods employed herein can also be such as those described in Harlow and Lane, (1988) supra. This can be accomplished, for example, by immunofluorescence techniques employing a fluorescently labeled antibody (see below) coupled with light microscopic, flow cytometric, or fluorimetric detection. The antibodies (or fragments thereof) useful in the present invention may, additionally, be employed histologically, as in immunofluorescence or immunoelectron microscopy, for in situ detection of the peptides or their allelic variants. In situ detection may be accomplished by removing a histological specimen from a patient, and applying thereto a labeled antibody of the present invention. The antibody (or fragment) is preferably applied by overlaying the labeled antibody (or fragment) onto a biological sample. Through the use of such a procedure, it is possible to determine not only the presence of the subject polypeptide, but also its distribution in the examined tissue. Using the present invention, one of ordinary skill will readily perceive that any of a wide variety of histological methods (such as staining procedures) can be modified in order to achieve such in situ detection.

Often a solid phase support or carrier is used as a support capable of binding an antigen or an antibody. Well-known supports or carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, gabbros, and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for the purposes of the present invention. The support material may have virtually any possible structural configuration so long as the coupled molecule is capable of binding to an antigen or antibody. Thus, the support configuration may be spherical, as in a bead, or cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, etc. or alternatively polystyrene beads. Those skilled in the art will know many other suitable carriers for binding antibody or antigen, or will be able to ascertain the same by use of routine experimentation.

Moreover, it will be understood that any of the above methods for detecting alterations in a gene or gene product or polymorphic variants can be used to monitor the course of treatment or therapy.

The methods described herein may be performed, for example, by utilizing pre-packaged diagnostic kits, such as those described below, comprising at least one probe or primer nucleic acid described herein, which may be conveniently used, e.g., to determine whether a subject has or is at risk of developing disease such as colorectal cancer.

Sample nucleic acid for use in the above-described diagnostic and prognostic methods can be obtained from any cell type or tissue of a subject. For example, a subject's bodily fluid (e.g. blood) can be obtained by known techniques (e.g., venipuncture). Alternatively, nucleic acid tests can be performed on dry samples (e.g., hair or skin). Fetal nucleic acid samples can be obtained from maternal blood as described in International Patent Application No. WO91/07660 to Bianchi. Alternatively, amniocytes or chorionic villi can be obtained for performing prenatal testing.

Diagnostic procedures can also be performed in situ directly upon tissue sections (fixed and/or frozen) of patient tissue obtained from biopsies or resections, such that no nucleic acid purification is necessary. Nucleic acid reagents can be used as probes and/or primers

for such in situ procedures (*see*, for example, Nuovo, G. J. (1992) "PCR *In Situ* Hybridization: Protocols And Applications", Raven Press, NY).

In addition to methods which focus primarily on the detection of one nucleic acid sequence, profiles can also be assessed in such detection schemes. Fingerprint profiles can be generated, for example, by utilizing a differential display procedure, Northern analysis and/or RT-PCR.

The invention described herein relates to methods and compositions for determining and identifying the allele present at the gene of interest's locus. This information is useful to diagnose and prognose disease progression as well as select the most effective treatment among treatment options. Probes can be used to directly determine the genotype of the sample or can be used simultaneously with or subsequent to amplification. The term "probes" includes naturally occurring or recombinant single- or double-stranded nucleic acids or chemically synthesized nucleic acids. They may be labeled by nick translation, Klenow fill-in reaction, PCR or other methods known in the art. Probes of the present invention, their preparation and/or labeling are described in Sambrook et al. (1989) supra. A probe can be a polynucleotide of any length suitable for selective hybridization to a nucleic acid containing a polymorphic region of the invention. Length of the probe used will depend, in part, on the nature of the assay used and the hybridization conditions employed.

In one embodiment of the invention, probes are labeled with two fluorescent dye molecules to form so-called "molecular beacons" (Tyagi, S. and Kramer, F.R. (1996) Nat. Biotechnol. **14**:303-8). Such molecular beacons signal binding to a complementary nucleic acid sequence through relief of intramolecular fluorescence quenching between dyes bound to opposing ends on an oligonucleotide probe. The use of molecular beacons for genotyping has been described (Kostrikis, L.G. (1998) Science **279**:1228-9) as has the use of multiple beacons simultaneously (Marras, S.A. (1999) Genet. Anal. **14**:151-6). A quenching molecule is useful with a particular fluorophore if it has sufficient spectral overlap to substantially inhibit fluorescence of the fluorophore when the two are held proixmal to one another, such as in a molecular beacon, or when attached to the ends of an oligonucleotide probe from about 1 to about 25 nucleotides.

Labeled probes also can be used in conjunction with amplification of a polymorphism. (Holland et al. (1991) Proc. Natl. Acad. Sci. **88:**7276-7280). U.S. Patent No. 5,210,015 by Gelfand et al. describe fluorescence-based approaches to provide real time measurements of amplification products during PCR. Such approaches have either employed intercalating dyes (such as ethidium bromide) to indicate the amount of double-stranded DNA present, or they have employed probes containing fluorescence-quencher pairs (also referred to as the "Taq-Man" approach) where the probe is cleaved during amplification to release a fluorescent molecule whose concentration is proportional to the amount of double-stranded DNA present. During amplification, the probe is digested by the nuclease activity of a polymerase when hybridized to the target sequence to cause the fluorescent molecule to be separated from the quencher molecule, thereby causing fluorescence from the reporter molecule to appear. The Taq-Man approach uses a probe containing a reporter molecule--quencher molecule pair that specifically anneals to a region of a target polynucleotide containing the polymorphism.

Probes can be affixed to surfaces for use as "gene chips." Such gene chips can be used to detect genetic variations by a number of techniques known to one of skill in the art. In one technique, oligonucleotides are arrayed on a gene chip for determining the DNA sequence of a by the sequencing by hybridization approach, such as that outlined in U.S. Patent Nos. 6,025,136 and 6,018,041. The probes of the invention also can be used for fluorescent detection of a genetic sequence. Such techniques have been described, for example, in U.S. Patent Nos. 5,968,740 and 5,858,659. A probe also can be affixed to an electrode surface for the electrochemical detection of nucleic acid sequences such as described by Kayem et al. U.S. Patent No. 5,952,172 and by Kelley, S.O. et al. (1999) Nucleic Acids Res. **27**:4830-4837.

Nucleic Acids

In one aspect, the nucleic acid sequences of the gene's allelic variants, or portions thereof, can be the basis for probes or primers, e.g., in methods for determining the identity of the allelic variant of the FcγRIIa 131 and/or FcγRIIIa 158 polymorphic region(s). Thus, they can be used in the methods of the invention to determine whether a subject is at risk of developing disease such as colorectal cancer or alternatively, which therapy is most likely to treat an individual's cancer.

The methods of the invention can use nucleic acids isolated from vertebrates. In one aspect, the vertebrate nucleic acids are mammalian nucleic acids. In a further aspect, the nucleic acids used in the methods of the invention are human nucleic acids.

Primers for use in the methods of the invention are nucleic acids which hybridize to a nucleic acid sequence which is adjacent to the region of interest or which covers the region of interest and is extended. A primer can be used alone in a detection method, or a primer can be used together with at least one other primer or probe in a detection method. Primers can also be used to amplify at least a portion of a nucleic acid. Probes for use in the methods of the invention are nucleic acids which hybridize to the region of interest and which are not further extended. For example, a probe is a nucleic acid which hybridizes to the polymorphic region of the gene of interest, and which by hybridization or absence of hybridization to the DNA of a subject will be indicative of the identity of the allelic variant of the polymorphic region of the gene of interest.

In one embodiment, primers comprise a nucleotide sequence which comprises a region having a nucleotide sequence which hybridizes under stringent conditions to about: 6, or alternatively 8, or alternatively 10, or alternatively 12, or alternatively 25, or alternatively 30, or alternatively 40, or alternatively 50, or alternatively 75 consecutive nucleotides of the gene of interest.

Primers can be complementary to nucleotide sequences located close to each other or further apart, depending on the use of the amplified DNA. For example, primers can be chosen such that they amplify DNA fragments of at least about 10 nucleotides or as much as several kilobases. Preferably, the primers of the invention will hybridize selectively to nucleotide sequences located about 150 to about 350 nucleotides apart.

For amplifying at least a portion of a nucleic acid, a forward primer (i.e., 5' primer) and a reverse primer (i.e., 3' primer) will preferably be used. Forward and reverse primers hybridize to complementary strands of a double stranded nucleic acid, such that upon extension from each primer, a double stranded nucleic acid is amplified.

Yet other preferred primers of the invention are nucleic acids which are capable of selectively hybridizing to an allelic variant of a polymorphic region of the gene of interest.

Thus, such primers can be specific for the gene of interest sequence, so long as they have a nucleotide sequence which is capable of hybridizing to the gene of interest.

The probe or primer may further comprises a label attached thereto, which, e.g., is capable of being detected, e.g. the label group is selected from amongst radioisotopes, fluorescent compounds, enzymes, and enzyme co-factors.

Additionally, the isolated nucleic acids used as probes or primers may be modified to become more stable. Exemplary nucleic acid molecules which are modified include phosphoramidate, phosphothioate and methylphosphonate analogs of DNA (see also U.S. Patent Nos. 5,176,996; 5,264,564 and 5,256,775).

The nucleic acids used in the methods of the invention can also be modified at the base moiety, sugar moiety, or phosphate backbone, for example, to improve stability of the molecule. The nucleic acids, e.g., probes or primers, may include other appended groups such as peptides (e.g., for targeting host cell receptors in vivo), or agents facilitating transport across the cell membrane. See, e.g., Letsinger et al., (1989) Proc. Natl. Acad. Sci. U.S.A. **86**:6553-6556; Lemaitre et al., (1987) Proc. Natl. Acad. Sci. **84**:648-652; and PCT Publication No. WO 88/09810, published Dec. 15, 1988), hybridization-triggered cleavage agents, (see, e.g., Krol et al., (1988) BioTechniques *6*:958-976) or intercalating agents (see, e.g., Zon (1988) Pharm. Res. **5**:539-549. To this end, the nucleic acid used in the methods of the invention may be conjugated to another molecule, e.g., a peptide, hybridization triggered cross-linking agent, transport agent, hybridization-triggered cleavage agent, etc.

The isolated nucleic acids used in the methods of the invention can also comprise at least one modified sugar moiety selected from the group including but not limited to arabinose, 2-fluoroarabinose, xylulose, and hexose or, alternatively, comprise at least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

The nucleic acids, or fragments thereof, to be used in the methods of the invention can be prepared according to methods known in the art and described, e.g., in Sambrook et al. (1989) supra. For example, discrete fragments of the DNA can be prepared and cloned

using restriction enzymes. Alternatively, discrete fragments can be prepared using the Polymerase Chain Reaction (PCR) using primers having an appropriate sequence under the manufacturer's conditions, (described above).

Oligonucleotides can be synthesized by standard methods known in the art, e.g. by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides can be synthesized by the method of Stein et al. (1988) Nucl. Acids Res. **16**:3209, methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports. Sarin et al. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**:7448-7451.

Methods of Treatment

The invention further provides methods of treating subjects having cancer selected from rectal cancer, colorectal cancer, (including metastatic CRC), colon cancer, gastric cancer, lung cancer (including non-small cell lung cancer) and esophageal cancer. In one embodiment, the method comprises (a) determining the identity of the allelic variant as identified herein; and (b) administering to the subject an effective amount of a compound or therapy (e.g., an anti-EGFR IgG1 antibody, mimetic or biological equivalent thereof). This therapy can be combined with other suitable therapies or treatments.

The antibodies and compositions are administered or delivered in an amount effective to treat the cancer and by any suitable means and with any suitable formulation as a composition and therefore includes a carrier such as a pharmaceutically acceptable carrier. Accordingly, a formulation comprising an antibody or biological equivalent thereof is further provided herein. The formulation can further comprise one or more preservative or stabilizer such as phenol, m-cresol, p-cresol, o-cresol, chlorocresol, benzyl alcohol, phenylmercuric nitrite, phenoxyethanol, formaldehyde, chlorobutanol, magnesium chloride (*e.g.*, hexahydrate), alkylparaben (methyl, ethyl, propyl, butyl and the like), benzalkonium chloride, benzethonium chloride, sodium dehydroacetate and thimerosal, or mixtures thereof in an aqueous diluent. Any suitable concentration or mixture can be used as known in the art, such as 0.001-5%, or any range or value therein, such as, but not limited to 0.001, 0.003, 0.005, 0.009, 0.01, 0.02, 0.03, 0.05, 0.09, 0.1, 0.2, 0.3, 0.4., 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5,

2.6, 2.7, 2.8, 2.9, 3.0, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.3, 4.5, 4.6, 4.7, 4.8, 4.9, or any range or value therein. Non-limiting examples include, no preservative, 0.1-2% m-cresol (*e.g.*, 0.2, 0.3. 0.4, 0.5, 0.9, 1.0%), 0.1-3% benzyl alcohol (*e.g.*, 0.5, 0.9, 1.1., 1.5, 1.9, 2.0, 2.5%), 0.001-0.5% thimerosal (*e.g.*, 0.005, 0.01), 0.001-2.0% phenol (*e.g.*, 0.05, 0.25, 0.28, 0.5, 0.9, 1.0%), 0.0005-1.0% alkylparaben(s) (*e.g.*, 0.00075, 0.0009, 0.001, 0.002, 0.005, 0.0075, 0.009, 0.01, 0.02, 0.05, 0.075, 0.09, 0.1, 0.2, 0.3, 0.5, 0.75, 0.9, and 1.0%).

The antibodies or biological equivalents thereof can be administered as a composition. A "composition" typically intends a combination of the active agent and another carrier, e.g., compound or composition, inert (for example, a detectable agent or label) or active, such as an adjuvant, diluent, binder, stabilizer, buffers, salts, lipophilic solvents, preservative, adjuvant or the like and include pharmaceutically acceptable carriers. Carriers also include pharmaceutical excipients and additives proteins, peptides, amino acids, lipids, and carbohydrates (*e.g.*, sugars, including monosaccharides, di-, tri-, tetra-, and oligosaccharides; derivatized sugars such as alditols, aldonic acids, esterified sugars and the like; and polysaccharides or sugar polymers), which can be present singly or in combination, comprising alone or in combination 1-99.99% by weight or volume. Exemplary protein excipients include serum albumin such as human serum albumin (HSA), recombinant human albumin (rHA), gelatin, casein, and the like. Representative amino acid/antibody components, which can also function in a buffering capacity, include alanine, glycine, arginine, betaine, histidine, glutamic acid, aspartic acid, cysteine, lysine, leucine, isoleucine, valine, methionine, phenylalanine, aspartame, and the like. Carbohydrate excipients are also intended within the scope of this invention, examples of which include but are not limited to monosaccharides such as fructose, maltose, galactose, glucose, D-mannose, sorbose, and the like; disaccharides, such as lactose, sucrose, trehalose, cellobiose, and the like; polysaccharides, such as raffinose, melezitose, maltodextrins, dextrans, starches, and the like; and alditols, such as mannitol, xylitol, maltitol, lactitol, xylitol sorbitol (glucitol) and myoinositol.

The term carrier further includes a buffer or a pH adjusting agent; typically, the buffer is a salt prepared from an organic acid or base. Representative buffers include organic acid salts such as salts of citric acid, ascorbic acid, gluconic acid, carbonic acid, tartaric acid,

succinic acid, acetic acid, or phthalic acid; Tris, tromethamine hydrochloride, or phosphate buffers. Additional carriers include polymeric excipients/additives such as polyvinylpyrrolidones, ficolls (a polymeric sugar), dextrates (e.g., cyclodextrins, such as 2-hydroxypropyl-.quadrature.-cyclodextrin), polyethylene glycols, flavoring agents, antimicrobial agents, sweeteners, antioxidants, antistatic agents, surfactants (e.g., polysorbates such as "TWEEN 20" and "TWEEN 80"), lipids (e.g., phospholipids, fatty acids), steroids (e.g., cholesterol), and chelating agents (e.g., EDTA).

As used herein, the term "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, and emulsions, such as an oil/water or water/oil emulsion, and various types of wetting agents. The compositions also can include stabilizers and preservatives and any of the above noted carriers with the additional provisio that they be acceptable for use in vivo. For examples of carriers, stabilizers and adjuvants, see Martin REMINGTON'S PHARM. SCI., 15th Ed. (Mack Publ. Co., Easton (1975) and Williams & Williams, (1995), and in the "PHYSICIAN'S DESK REFERENCE", 52nd ed., Medical Economics, Montvale, N.J. (1998).

An "effective amount" is an amount sufficient to effect beneficial or desired results. An effective amount can be administered in one or more administrations, applications or dosages.

The invention provides an article of manufacture, comprising packaging material and at least one vial comprising a solution of at least one antibody or its biological equivalent with the prescribed buffers and/or preservatives, optionally in an aqueous diluent, wherein said packaging material comprises a label that indicates that such solution can be held over a period of 1, 2, 3, 4, 5, 6, 9, 12, 18, 20, 24, 30, 36,40, 48, 54, 60, 66, 72 hours or greater. The invention further comprises an article of manufacture, comprising packaging material, a first vial comprising at least one lyophilized antibody or its biological equivalent and a second vial comprising an aqueous diluent of prescribed buffer or preservative, wherein said packaging material comprises a label that instructs a patient to reconstitute the therapeutic in the aqueous diluent to form a solution that can be held over a period of twenty-four hours or greater.

The antibody or equivalent thereof is prepared to a concentration includes amounts yielding upon reconstitution, if in a wet/dry system, concentrations from about 1.0 μg/ml to about 1000 mg/ml, although lower and higher concentrations are operable and are dependent on the intended delivery vehicle, e.g., solution formulations will differ from transdermal patch, pulmonary, transmucosal, or osmotic or micro pump methods.

The formulations of the present invention can be prepared by a process which comprises mixing at least one antibody or biological equivalent and a preservative selected from the group consisting of phenol, m-cresol, p-cresol, o-cresol, chlorocresol, benzyl alcohol, alkylparaben, (methyl, ethyl, propyl, butyl and the like), benzalkonium chloride, benzethonium chloride, sodium dehydroacetate and thimerosal or mixtures thereof in an aqueous diluent. Mixing of the antibody and preservative in an aqueous diluent is carried out using conventional dissolution and mixing procedures. For example, a measured amount of at least one antibody in buffered solution is combined with the desired preservative in a buffered solution in quantities sufficient to provide the antibody and preservative at the desired concentrations. Variations of this process would be recognized by one of skill in the art, e.g., the order the components are added, whether additional additives are used, the temperature and pH at which the formulation is prepared, are all factors that can be optimized for the concentration and means of administration used.

The compositions and formulations can be provided to patients as clear solutions or as dual vials comprising a vial of lyophilized antibody that is reconstituted with a second vial containing the aqueous diluent. Either a single solution vial or dual vial requiring reconstitution can be reused multiple times and can suffice for a single or multiple cycles of patient treatment and thus provides a more convenient treatment regimen than currently available. Recognized devices comprising these single vial systems include those pen-injector devices for delivery of a solution such as BD Pens, BD Autojectore, Humaject.RTM.' NovoPen.RTM., B-D.RTM.Pen, AutoPen.RTM., and OptiPen.RTM., GenotropinPen.RTM., Genotronorm Pen.RTM., Humatro Pen.RTM., Reco-Pen.RTM., Roferon Pen.RTM., Biojector.RTM., iject.RTM., J-tip Needle-Free Injector.RTM., Intraject.RTM., Medi-Ject.RTM., *e.g.*, as made or developed by Becton Dickensen (Franklin Lakes, N.J. available at bectondickenson.com), Disetronic (Burgdorf, Switzerland, available at disetronic.com; Bioject, Portland, Oregon (available at

bioject.com); National Medical Products, Weston Medical (Peterborough, UK, available at weston-medical.com), Medi-Ject Corp (Minneapolis, Minn., available at mediject.com).

Various delivery systems are known and can be used to administer a therapeutic agent of the invention, e.g., encapsulation in liposomes, microparticles, microcapsules, expression by recombinant cells, receptor-mediated endocytosis. See e.g., Wu and Wu (1987) J. Biol. Chem. **262**:4429-4432 for construction of a therapeutic nucleic acid as part of a retroviral or other vector, etc. Methods of delivery include but are not limited to intra-arterial, intra-muscular, intravenous, intranasal and oral routes. In a specific embodiment, it may be desirable to administer the pharmaceutical compositions of the invention locally to the area in need of treatment; this may be achieved by, for example, and not by way of limitation, local infusion during surgery, by injection or by means of a catheter.

The agents identified herein as effective for their intended purpose can be administered to subjects or individuals identified by the methods herein as suitable for the therapy, Therapeutic amounts can be empirically determined and will vary with the pathology being treated, the subject being treated and the efficacy and toxicity of the agent.

Biological Equivalent Antibodies and Therapies

In one aspect, after determining that antibody therapy alone or in combination with other suitable therapy is likely to provide a benefit to the patient, the invention further comprises administration of an antibody, fragment, variant or derivative thereof that binds EGFR such as Cetuximab. The antibodies of this invention are monoclonal antibodies, although in certain aspects, polyclonal antibodies can be utilized. They also can be EGFR-neutralizing functional fragments, antibody derivatives or antibody variants. They can be chimeric, humanized, or totally human. A functional fragment of an antibody includes but is not limited to Fab, Fab', Fab2, Fab'2, and single chain variable regions. Antibodies can be produced in cell culture, in phage, or in various animals, including but not limited to cows, rabbits, goats, mice, rats, hamsters, guinea pigs, sheep, dogs, cats, monkeys, chimpanzees, apes, etc. So long as the fragment or derivative retains specificity of binding or neutralization ability as the antibodies of this invention it can be used. Antibodies can be tested for specificity of binding by comparing binding to appropriate antigen to binding to irrelevant antigen or antigen mixture under a given set of conditions. If the antibody

binds to the appropriate antigen at least 2, 5, 7, and preferably 10 times more than to irrelevant antigen or antigen mixture then it is considered to be specific.

The antibodies also are characterized by their ability to specifically bind to an EGFR epitope. The monoclonal antibodies of the invention can be generated using conventional hybridoma techniques known in the art and well-described in the literature. For example, a hybridoma is produced by fusing a suitable immortal cell line (e.g., a myeloma cell line such as, but not limited to, Sp2/0, Sp2/0-AG14, NSO, NS1, NS2, AE-1, L.5, >243, P3X63Ag8.653, Sp2 SA3, Sp2 MAI, Sp2 SS1, Sp2 SA5, U397, MLA 144, ACT IV, MOLT4, DA-1, JURKAT, WEHI, K-562, COS, RAJI, NIH 3T3, HL-60, MLA 144, NAMAIWA, NEURO 2A, CHO, PerC.6, YB2/O) or the like, or heteromyelomas, fusion products thereof, or any cell or fusion cell derived there from, or any other suitable cell line as known in the art (see, e.g., www.atcc.org, www.lifetech.com., and the like), with antibody producing cells, such as, but not limited to, isolated or cloned spleen, peripheral blood, lymph, tonsil, or other immune or B cell containing cells, or any other cells expressing heavy or light chain constant or variable or framework or CDR sequences, either as endogenous or heterologous nucleic acid, as recombinant or endogenous, viral, bacterial, algal, prokaryotic, amphibian, insect, reptilian, fish, mammalian, rodent, equine, ovine, goat, sheep, primate, eukaryotic, genomic DNA, cDNA, rDNA, mitochondrial DNA or RNA, chloroplast DNA or RNA, hnRNA, mRNA, tRNA, single, double or triple stranded, hybridized, and the like or any combination thereof. Antibody producing cells can also be obtained from the peripheral blood or, preferably the spleen or lymph nodes, of humans or other suitable animals that have been immunized with the antigen of interest. Any other suitable host cell can also be used for expressing-heterologous or endogenous nucleic acid encoding an antibody, specified fragment or variant thereof, of the present invention. The fused cells (hybridomas) or recombinant cells can be isolated using selective culture conditions or other suitable known methods, and cloned by limiting dilution or cell sorting, or other known methods.

Other suitable methods of producing or isolating antibodies of the requisite specificity can be used, including, but not limited to, methods that select recombinant antibody from a peptide or protein library (e.g., but not limited to, a bacteriophage, ribosome, oligonucleotide, RNA, cDNA, or the like, display library; e.g., as available from various

commercial vendors such as Cambridge Antibody Technologies (Cambridgeshire, UK), MorphoSys (Martinsreid/Planegg, Del.), Biovation (Aberdeen, Scotland, UK) BioInvent (Lund, Sweden), using methods known in the art. See U.S. Pat. Nos. 4,704,692; 5,723,323; 5,763,192; 5,814,476; 5,817,483; 5,824,514; 5,976,862. Alternative methods rely upon immunization of transgenic animals (e.g., SCID mice, Nguyen et al. (1977) Microbiol. Immunol. **41**:901-907 (1997); Sandhu et al., (1996) Crit. Rev. Biotechnol. **16**:95-118; Eren et al. (1998) Immunol. **93**:154-161 that are capable of producing a repertoire of human antibodies, as known in the art and/or as described herein. Such techniques, include, but are not limited to, ribosome display (Hanes et al. (1997) Proc. Natl. Acad. Sci. USA, **94**:4937-4942; Hanes et al., (1998) Proc. Natl. Acad. Sci. USA, **95**:14130-14135); single cell antibody producing technologies (e.g., selected lymphocyte antibody method ("SLAM") (U.S. Pat. No. 5,627,052, Wen et al. (1987) J. Immunol. **17**:887-892; Babcook et al., Proc. Natl. Acad. Sci. USA (1996) **93**:7843-7848); gel microdroplet and flow cytometry (Powell et al. (1990) Biotechnol. **8**:333-337; One Cell Systems, (Cambridge, Mass).; Gray et al. (1995) J. Imm. Meth. **182**:155-163; Kenny et al. (1995) Bio/Technol. **13**:787-790); B-cell selection (Steenbakkers et al. (1994) Molec. Biol. Reports **19**:125-134 (1994).

Antibody variants of the present invention can also be prepared using delivering a polynucleotide encoding an antibody of this invention to a suitable host such as to provide transgenic animals or mammals, such as goats, cows, horses, sheep, and the like, that produce such antibodies in their milk. These methods are known in the art and are described for example in U.S. Pat. Nos. 5,827,690; 5,849,992; 4,873,316; 5,849,992; 5,994,616; 5,565,362; and 5,304,489.

The term "antibody variant" includes post-translational modification to linear polypeptide sequence of the antibody or fragment. For example, U.S. Patent No. 6,602,684 B1 describes a method for the generation of modified glycol-forms of antibodies, including whole antibody molecules, antibody fragments, or fusion proteins that include a region equivalent to the Fc region of an immunoglobulin, having enhanced Fc-mediated cellular toxicity, and glycoproteins so generated.

Antibody variants also can be prepared by delivering a polynucleotide of this invention to provide transgenic plants and cultured plant cells (e.g., but not limited to tobacco, maize,

and duckweed) that produce such antibodies, specified portions or variants in the plant parts or in cells cultured there from. For example, Cramer et al. (1999) Curr. Top. Microbol. Immunol. **240**:95-118 and references cited therein, describe the production of transgenic tobacco leaves expressing large amounts of recombinant proteins, e.g., using an inducible promoter. Transgenic maize have been used to express mammalian proteins at commercial production levels, with biological activities equivalent to those produced in other recombinant systems or purified from natural sources. See, e.g., Hood et al. (1999) Adv. Exp. Med. Biol. **464**:127-147 and references cited therein. Antibody variants have also been produced in large amounts from transgenic plant seeds including antibody fragments, such as single chain antibodies (scFv's), including tobacco seeds and potato tubers. See, e.g., Conrad et al.(1998) Plant Mol. Biol. **38**:101-109 and reference cited therein. Thus, antibodies of the present invention can also be produced using transgenic plants, according to know methods.

Antibody derivatives can be produced, for example, by adding exogenous sequences to modify immunogenicity or reduce, enhance or modify binding, affinity, on-rate, off-rate, avidity, specificity, half-life, or any other suitable characteristic. Generally part or all of the non-human or human CDR sequences are maintained while the non-human sequences of the variable and constant regions are replaced with human or other amino acids.

In general, the CDR residues are directly and most substantially involved in influencing antigen binding. Humanization or engineering of antibodies of the present invention can be performed using any known method, such as but not limited to those described in U.S. Pat. Nos. 5,723,323, 5,976,862, 5,824,514, 5,817,483, 5,814,476, 5,763,192, 5,723,323, 5,766,886, 5,714,352, 6,204,023, 6,180,370, 5,693,762, 5,530,101, 5,585,089, 5,225,539; and 4,816,567.

Techniques for making partially to fully human antibodies are known in the art and any such techniques can be used. According to one embodiment, fully human antibody sequences are made in a transgenic mouse which has been engineered to express human heavy and light chain antibody genes. Multiple strains of such transgenic mice have been made which can produce different classes of antibodies. B cells from transgenic mice which are producing a desirable antibody can be fused to make hybridoma cell lines for continuous production of the desired antibody. See for example, Russel, N.D. et al. (2000)

Infection and Immunity April:1820-1826; Gallo, M. L. et al. (2000) European J. of Immun. **30**:534-540; Green, L. L. (1999) J. of Immun. Methods **231**:11-23; Yang, X-D et al. (1999A) J. of Leukocyte Biology **66**:401-410; Yang, X-D (1999B) Cancer Research **59**(6):1236-1243; Jakobovits, A. (1998) Advanced Drug Delivery Reviews **31**:33-42; Green, L. and Jakobovits, A. (1998) J. Exp. Med. **188**(3):483-495; Jakobovits, A. (1998) Exp. Opin. Invest. Drugs **7**(4):607-614; Tsuda, H. et al. (1997) Genomics **42**:413-421; Sherman-Gold, R. (1997) Genetic Engineering News **17**(14); Mendez, M. et al. (1997) Nature Genetics **15**:146-156; Jakobovits, A. (1996) Weir's Handbook of Experimental Immunology, The Integrated Immune System Vol. IV, 194.1-194.7; Jakobovits, A. (1995) Current Opinion in Biotechnology **6**:561-566; Mendez, M. et al. (1995) Genomics **26**:294-307; Jakobovits, A. (1994) Current Biology **4**(8):761-763; Arbones, M. et al. (1994) Immunity **1**(4):247-260; Jakobovits, A. (1993) Nature **362**(6417):255-258; Jakobovits, A. et al. (1993) Proc. Natl. Acad. Sci. USA **90**(6):2551-2555; Kucherlapati, et al. U.S. Patent No. 6,075,181.

Human monoclonal antibodies can also be produced by a hybridoma which includes a B cell obtained from a transgenic nonhuman animal, e.g., a transgenic mouse, having a genome comprising a human heavy chain transgene and a light chain transgene fused to an immortalized cell.

The antibodies of this invention also can be modified to create chimeric antibodies. Chimeric antibodies are those in which the various domains of the antibodies' heavy and light chains are coded for by DNA from more than one species. See, e.g., U.S. Patent No.: 4,816,567.

The term "antibody derivative" also includes "diabodies" which are small antibody fragments with two antigen-binding sites, wherein fragments comprise a heavy chain variable domain (V) connected to a light chain variable domain (V) in the same polypeptide chain (VH V). See for example, EP 404,097; WO 93/11161; and Hollinger et al., (1993) Proc. Natl. Acad. Sci. USA **90**:6444-6448. By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. See also, U.S. Patent No. 6,632,926 to Chen et al. which discloses antibody variants that have one or more amino acids inserted into a hypervariable region of the parent antibody

and a binding affinity for a target antigen which is at least about two fold stronger than the binding affinity of the parent antibody for the antigen.

The term "antibody derivative" further includes "linear antibodies". The procedure for making the is known in the art and described in Zapata et al. (1995) Protein Eng. 8(10):1057-1062. Briefly, these antibodies comprise a pair of tandem Fd segments (V-C 1-VH -C1) which form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

The antibodies of this invention can be recovered and purified from recombinant cell cultures by known methods including, but not limited to, protein A purification, ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. High performance liquid chromatography ("HPLC") can also be used for purification.

Antibodies of the present invention include naturally purified products, products of chemical synthetic procedures, and products produced by recombinant techniques from a eukaryotic host, including, for example, yeast, higher plant, insect and mammalian cells, or alternatively from a prokaryotic cells as described above.

Antibodies can also be conjugated, for example, to a pharmaceutical agent, such as chemotherapeutic drug or a toxin. They can be linked to a cytokine, to a ligand, to another antibody. Suitable agents for coupling to antibodies to achieve an anti-tumor effect include cytokines, such as interleukin 2 (IL-2) and Tumor Necrosis Factor (TNF); photosensitizers, for use in photodynamic therapy, including aluminum (III) phthalocyanine tetrasulfonate, hematoporphyrin, and phthalocyanine; radionuclides, such as iodine-131 (^{131}I), yttrium-90 (^{90}Y), bismuth-212 (^{212}Bi), bismuth-213 (^{213}Bi), technetium-99m (^{99m}Tc), rhenium-186 (^{186}Re), and rhenium-188 (^{188}Re); antibiotics, such as doxorubicin, adriamycin, daunorubicin, methotrexate, daunomycin, neocarzinostatin, and carboplatin; bacterial, plant, and other toxins, such as diphtheria toxin, pseudomonas exotoxin A, staphylococcal enterotoxin A, abrin-A toxin, ricin A (deglycosylated ricin A and native ricin A), TGF-alpha toxin, cytotoxin from chinese cobra (naja naja atra), and

gelonin (a plant toxin); ribosome inactivating proteins from plants, bacteria and fungi, such as restrictocin (a ribosome inactivating protein produced by *Aspergillus restrictus*), saporin (a ribosome inactivating protein from *Saponaria officinalis*), and RNase; tyrosine kinase inhibitors; ly207702 (a difluorinated purine nucleoside); liposomes containing anti cystic agents (e.g., antisense oligonucleotides, plasmids which encode for toxins, methotrexate, etc.); and other antibodies or antibody fragments, such as F(ab).

Antibodies can also be used in immunohistochemical assays to detect the presence or expression level of a protein of interest. They are further useful to detect the presence or absence of EGFR in a patient sample. In these and other aspects of this invention, it will be useful to detectably or therapeutically label the antibody. Methods for conjugating antibodies to these agents are known in the art. For the purpose of illustration only, antibodies can be labeled with a detectable moiety such as a radioactive atom, a chromophore, a fluorophore, or the like. With respect to preparations containing antibodies covalently linked to organic molecules, they can be prepared using suitable methods, such as by reaction with one or more modifying agents. Examples of such include modifying and activating groups. A "modifying agent" as the term is used herein, refers to a suitable organic group (e.g., hydrophilic polymer, a fatty acid, a fatty acid ester) that comprises an activating group. Specific examples of these are provided supra. An "activating group" is a chemical moiety or functional group that can, under appropriate conditions, react with a second chemical group thereby forming a covalent bond between the modifying agent and the second chemical group. Examples of such are electrophilic groups such as tosylate, mesylate, halo (chloro, bromo, fluoro, iodo), N-hydroxysuccinimidyl esters (NHS), and the like. Activating groups that can react with thiols include, for example, maleimide, iodoacetyl, acrylolyl, pyridyl disulfides, 5-thiol-2-nitrobenzoic acid thiol (TNB-thiol), and the like. An aldehyde functional group can be coupled to amine- or hydrazide-containing molecules, and an azide group can react with a trivalent phosphorous group to form phosphoramidate or phosphorimide linkages. Suitable methods to introduce activating groups into molecules are known in the art (see for example, Hermanson, G. T., BIOCONJUGATE TECHNIQUES, Academic Press: San Diego, Calif. (1996)). An activating group can be bonded directly to the organic group (e.g., hydrophilic polymer, fatty acid, fatty acid ester), or through a linker moiety, for example a divalent C_1-C_{12} group wherein one or more carbon atoms can be replaced by a

heteroatom such as oxygen, nitrogen or sulfur. Suitable linker moieties include, for example, tetraethylene glycol. Modifying agents that comprise a linker moiety can be produced, for example, by reacting a mono-Boc-alkyldiamine (e.g., mono-Boc-ethylenediamine, mono-Boc-diaminohexane) with a fatty acid in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) to form an amide bond between the free amine and the fatty acid carboxylate. The Boc protecting group can be removed from the product by treatment with trifluoroacetic acid (TFA) to expose a primary amine that can be coupled to another carboxylate as described, or can be reacted with maleic anhydride and the resulting product cyclized to produce an activated maleimido derivative of the fatty acid.

The modified antibodies of the invention can be produced by reacting a human antibody or antigen-binding fragment with a modifying agent. For example, the organic moieties can be bonded to the antibody in a non-site specific manner by employing an amine-reactive modifying agent, for example, an NHS ester of PEG. Modified human antibodies or antigen-binding fragments can also be prepared by reducing disulfide bonds (e.g., intra-chain disulfide bonds) of an antibody or antigen-binding fragment. The reduced antibody or antigen-binding fragment can then be reacted with a thiol-reactive modifying agent to produce the modified antibody of the invention. Modified human antibodies and antigen-binding fragments comprising an organic moiety that is bonded to specific sites of an antibody of the present invention can be prepared using suitable methods, such as reverse proteolysis. See generally, Hermanson, G. T., BIOCONJUGATE TECHNIQUES, Academic Press: San Diego, Calif. (1996).

Kits

As set forth herein, the invention provides diagnostic methods for determining the type of allelic variant of a polymorphic region present in the gene of interest or the expression level of a gene of interest. In some embodiments, the methods use probes or primers comprising nucleotide sequences which are complementary to the polymorphic region of the gene of interest. Accordingly, the invention provides kits for performing these methods.

In an embodiment, the invention provides a kit for determining whether a subject responds to cancer treatment or alternatively one of various treatment options. The kits contain one of more of the compositions described above and instructions for use. As an example only, the invention also provides kits for determining response to cancer treatment containing a first and a second oligonucleotide specific for the polymorphic region of the gene. Oligonucleotides "specific for" a genetic locus bind either to the polymorphic region of the locus or bind adjacent to the polymorphic region of the locus. For oligonucleotides that are to be used as primers for amplification, primers are adjacent if they are sufficiently close to be used to produce a polynucleotide comprising the polymorphic region. In one embodiment, oligonucleotides are adjacent if they bind within about 1-2 kb, and preferably less than 1 kb from the polymorphism. Specific oligonucleotides are capable of hybridizing to a sequence, and under suitable conditions will not bind to a sequence differing by a single nucleotide.

The kit can comprise at least one probe or primer which is capable of specifically hybridizing to the polymorphic region of the gene of interest and instructions for use. The kits preferably comprise at least one of the above described nucleic acids. Preferred kits for amplifying at least a portion of the gene of interest comprise two primers, at least one of which is capable of hybridizing to the allelic variant sequence. Such kits are suitable for detection of genotype by, for example, fluorescence detection, by electrochemical detection, or by other detection.

Oligonucleotides, whether used as probes or primers, contained in a kit can be detectably labeled. Labels can be detected either directly, for example for fluorescent labels, or indirectly. Indirect detection can include any detection method known to one of skill in the art, including biotin-avidin interactions, antibody binding and the like. Fluorescently labeled oligonucleotides also can contain a quenching molecule. Oligonucleotides can be bound to a surface. In one embodiment, the preferred surface is silica or glass. In another embodiment, the surface is a metal electrode.

Yet other kits of the invention comprise at least one reagent necessary to perform the assay. For example, the kit can comprise an enzyme. Alternatively the kit can comprise a buffer or any other necessary reagent.

Conditions for incubating a nucleic acid probe with a test sample depend on the format employed in the assay, the detection methods used, and the type and nature of the nucleic acid probe used in the assay. One skilled in the art will recognize that any one of the commonly available hybridization, amplification or immunological assay formats can readily be adapted to employ the nucleic acid probes for use in the present invention. Examples of such assays can be found in Chard, T. (1986) "An Introduction to Radioimmunoassay and Related Techniques" Elsevier Science Publishers, Amsterdam, The Netherlands ; Bullock, G.R. et al., "Techniques in Immunocytochemistry" Academic Press, Orlando, FL Vol. 1 (1982), Vol. 2 (1983), Vol. 3 (1985); Tijssen, P., (1985) "Practice and Theory of Immunoassays: Laboratory Techniques in Biochemistry and Molecular Biology", Elsevier Science Publishers, Amsterdam, The Netherlands.

The test samples used in the diagnostic kits include cells, protein or membrane extracts of cells, or biological fluids such as sputum, blood, serum, plasma, or urine. The test sample used in the above-described method will vary based on the assay format, nature of the detection method and the tissues, cells or extracts used as the sample to be assayed. Methods for preparing protein extracts or membrane extracts of cells are known in the art and can be readily adapted in order to obtain a sample which is compatible with the system utilized.

The kits can include all or some of the positive controls, negative controls, reagents, primers, sequencing markers, probes and antibodies described herein for determining the subject's genotype in the polymorphic region of the gene of interest.

As amenable, these suggested kit components may be packaged in a manner customary for use by those of skill in the art. For example, these suggested kit components may be provided in solution or as a liquid dispersion or the like.

Other Uses for the Nucleic Acids of the Invention

The identification of the allele of the gene of interest can also be useful for identifying an individual among other individuals from the same species. For example, DNA sequences can be used as a fingerprint for detection of different individuals within the same species. Thompson, J. S. and Thompson, eds., (1991) "Genetics in Medicine", W B Saunders Co., Philadelphia, Pa. This is useful, e.g., in forensic studies.

The invention now being generally described, it will be more readily understood by reference to the following examples which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention.

EXPERIMENTAL EXAMPLES

Example 1

The use of the EGFR targeting monoclonal antibody Cetuximab in patients with metastatic colorectal cancer is demonstrating promising efficacy in different phase II clinical trials. However, until now, there are no reliable markers to identify patients who will most likely benefit from this therapy. Clinical trials have failed to show a significant correlation between EGFR expression based on immunhistochemistry (IHC) and response to treatment with either cetuximab and CPT-11 or cetuximab alone. Reported in Chung and Saltz (2005) supra.

Cetuximab is a IGg1 antibody it is able to generate an antibody mediated cell cytotoxicity. Recent data have shown that a polymorphisms in the FC gamma was associated with efficacy of Rituximab in patients with hematological malignancies. Miescher, S. et al. (2004) supra.

The patients were from the USC/Norris Comprehensive Cancer Center, Los Angeles, who took part in a II open-label multi-center study (IMCL-0144) of Cetuximab. All 35 patients signed an additional informed consent for blood collection to study molecular correlates. The patients had histopathologically confirmed metastatic CRC who failed CPT-11/5-FU/LV and oxaliplatin therapy provided the patient progressed within 6 months of completing adjuvant therapy. The study was investigated at USC/Norris Comprehensive Cancer Center and approved by the Institutional Review Board of the University. All patients had immunhistochemical evidence of EGFR expression in their tumor samples.

Patients were treated with Cetuximab at standard loading dose 400 mg/m^2 over 2 hours, followed by weekly 250 mg/m^2 treatment over 1 hour. Treatment was continued until progression of disease or toxicity occurred and patients were evaluated every 6 weeks for tumor response.

For the purpose of illustration only, peripheral blood sample can be collected from each patient, and genomic DNA can be extracted from white blood cells using the QiaAmp kit (Qiagen, Valencia, CA). Polymorphisms in the FcγRIIa and FcγRIIIa gene were all tested using methods well known in the art, e.g., as described in Weng and Levy (2003) J. Clin. Oncol. **21**:3940-3947, Carton et al. (2002) Blood **99(3)**:754-758 and Koene, H.R. et al. (1997) Blood **90(3)**:1109-1114.

Polymorphisms in the FcγIIa were associated with time to tumor progression (p=0.037) and response was borderline (p=0.082).

The 131 H/R polymorphism was tested in 35 advanced colorectal cancer patients treated with single agent Cetuximab. Patients with FcγRIIa H/H or H/R genotype showed better time to progression (p=0.037,log-rank test) and overall survival compared to patients with R/R genotype (p=0.22, log-rank test). Also, there was a trend significance in tumor response when patients with R/R genotype were compared with patients with H/H or H/R genotype (p=0.08, fisher exact test). See Figure 1.

Experiment 2

In an extension of the study reported in Experiment 1, thirty-nine patients with metastatic colorectal cancer who failed at least two prior chemotherapy (both CPT-11 and Oxaliplatin) were enrolled at the University of Southern California/Norris Comprehensive Cancer Center, Los Angeles between October 2002 and March of 2003. These patients took in part in a phase II single agent Cetuximab treatment clinical trial (IMCL-0144) including 346 patients. This study was investigated at USC/Norris Comprehensive Cancer Center and approved by the Institutional Review Board of the University of Southern California for Medical Sciences. All patients had immunhistochemical evidence of EGFR expression in their tumor samples. Patients were treated with Cetuximab at standard doses 400 mg/m^2 loading dose over 2 hours, then 250 mg/m^2 over 1 hour weekly.

A peripheral blood sample was collected from each patient at the beginning of treatment start and genomic DNA was extracted from white blood cells using QiaAmp kit (Qiagen, Valencia, CA). FcγRIIIa V158F polymorphism, FcγRIIa 131 H/R polymorphism, was done by PCR-RFLP method. See Jiang et al. (1996) J Immunol Methods **199**: 55-59, for a description of this method.

The results are shown in Table 1 and Figure 2. The reported data show that two immunoglobulin G Fragment C Receptor polymorphisms, FcγRIIIa 158V/F and FcγRIIa 131 H/R are molecular markers for clinical outcome of the EGFR-expressing refractory metastatic colorectal caner patients treated with single agent EGFR inhibitor Cetuximab. This data also demonstrated that ADCC may have clinical significance in patients treated with Cetuximab.

Thus, this invention provides a method for selecting a therapeutic regimen for treating cancer in a patient, the method comprising identifying the genotype of a patient at the FcγRIIa 131 position. Patients with an H allele (i.e., H/H or H/R) polymorphism are more stable and show a partial response when treated with Cetuximab. Patients with a F allele (F/F or F/V) also show a partial response or were more stable over the course of the study. Stated another way, patients either 131 R/R or alternatively 158 V/V were less likely to respond to Cetuximab therapy as evidenced by no response to treatment or disease progression. Dual analysis showed that patients 131 H and 158 F were more stable (little or no disease progression) even though a partial response was not significantly different than patients 131 R/R and 158 V/V.

Thus, the invention provides a method for selecting a therapeutic regimen for treating a cancer in a patient expressing EGFR, the method comprising identifying the FcγRIIa 131 and/or FcγRIIIa 158 genomic polymorphism or genotype that is correlative to treatment outcome of the cancer in the patient. In one aspect, the cancer is treatable by the administration of a chemotherapeutic drug or agent selected from the group: a small molecule fluoropyrimidine, a platinum drug, a topoisomerase inhibitor and an anti-EGFR IgG1 antibody or a biological equivalent thereof.

In another aspect, the cancer is selected from the group consisting of colon cancer, rectal cancer, CRC, metastatic CRC, esophageal cancer, gastric cancer, lung cancer and non-small cell lung cancer.

In another aspect, the cancer treatment further comprises radiation therapy which can combined with chemotherapy. Suitable chemotherapies may include, but are not limited to Cetuximab, CPT-11, 5-fluorouracil (5-FU), LV and oxalplatinum. In another aspect, the treatment specifically excludes one or more of the members of this group.

The method will identify those cancers suitably treated by an IgG1 antibody, mimetic or equivalent, e.g, anti-EGFR IgG1 antibody which comprises an active fragment or variant of Cetuximab antibody.

The above noted method for determining the identity of the FcγRIIa and/or FcγRIIIa polymorphism also is predictive of the survival time or stable disease for a patient with a cancer identified above after treatment with an anti-EGFR IgG1 antibody, mimetic or equivalent. Such anti-EGFR IgG1 antibody can be Cetuximab or a molecule which comprises an active fragment or variant of Cetuximab antibody or biological equivalent thereof.

It is to be understood that while the invention has been described in conjunction with the above embodiments, that the foregoing description and examples are intended to illustrate and not limit the scope of the invention. Other aspects, advantages and modifications within the scope of the invention will be apparent to those skilled in the art to which the invention pertains. Several aspects of the invention are listed below.

Table 1

FCGR polymorphisms and clinical outcome among patients in protocol 3C-02-3

		Response			Toxicity		Progression-Free survival		Overall Survival	
	N	PR	SD	PD	Grade 0-1	Grade 2-3	Median, Mo (95%CI)	Relative Risk (95% CI)	Median, Mo (95%CI)	Relative Risk (95% CI)
FCGR2A										
H/H	9	0 (0%)	7 (78%)	2 (22%)	3 (33%)	6 (67%)	2.4 (2.4, 3.7)	1 (Reference)	4.5 (4.4, 8.7)	1 (Reference)
H/R	17	1 (6%)	12 (71%)	4 (24%)	7 (41%)	10 (59%)	3.7 (2.0, 5.0)	0.56 (0.23-1.32)	12.0 (3.4, 15.4)	0:52 (0.20-1.37)
R/R	9	1 (14%)	0 (0%)	6 (86%)	5 (56%)	4 (44%)	1.1 (1.0, 1.4)	1.43 (0.53-3.86)	2.3 (2.1, 8.5)	0.84 (0.29-2.41)
P value			0.082			0.75		0.037		0.22
FCGR3A										
F/F	16	2 (14%)	8 (57%)	4 (29%)	7 (44%)	9 (56%)	2.3 (1.2, 3.7)	1 (Reference)	5.5 (2.2, 15.0)	1 (Reference)
F/V	14	0 (0%)	10 (71%)	4 (29%)	5 (36%)	9 (64%)	2.4 (1.4, 4.6)	0.72 (0.34-1.53)	10.7 (4.4, 15.4)	0.80 (0.38-1.71)
V/V	5	0 (0%)	1 (20%)	4 (80%)	3 (60%)	2 (40%)	1.1 (0.7, 3.7)	2.28 (0.78-6.63)	3.4 (1.4, 8.7)	1.98 (0.67-5.86)
P value			0.067			0.66		0.055		0.19
FCGR combined										
H and F	22	1 (5%)	18 (82%)	3 (14%)	8 (36%)	14 (64%)	3.7 (2.4, 4.4)	1 (Reference)	10.7 (4.8, 15.2)	1 (Reference)
R/R or V/V	13	1 (9%)	1 (9%)	9 (82%)	7 (54%)	6 (46%)	1.1 (1.0, 1.4)	1.78 (0.87-3.66)	2.3 (2.1, 8.5)	1.78 (0.87-3.66)
P value			<0.001			0.48		0.004		0.093

* *P* values were based on the exact Jonckheere-Terpstra test for response, Fisher's exact test for toxicity, and log-rank test for time-to-event variables. PR: Partial Response, SD: Stable Disease, PD: Progressive Disease

What is claimed is:

1. A method for identifying responsiveness to anti-EGFR therapy for a patient having cells expressing epidermal growth factor receptor (EGFR), comprising determining in a sample from said patient at least one allelic pattern selected from the FcγRIIa gene at position 131 and the FcγRIIIa gene at position 158, wherein the presence of at least one H at position 131 of FcγRIIa or at least one F at position 158 of FcγRIIIa, in said sample identifies responsiveness to said therapy for said patient.

2. The method of claim 1, wherein the allelic pattern from said patient sample is H at position 131 of FcγRIIa and F at amino acid position 158 of FcγRIIIa

3. The method of claim 1, wherein the anti-EGFR therapy is anti-EGFR IgG 1 antibody therapy.

4. The method of claim 2, wherein the anti-EGFR antibody is Cetuximab.

5. The method of claim 1, wherein the cancer is selected from rectal cancer, colorectal cancer, metastatic colorectal cancer, colon cancer, gastric cancer, lung cancer, non-small cell lung cancer and esophageal cancer.

6. The method of claim 1, wherein the sample comprises tumor tissue, normal tissue adjacent to said tumor, normal tissue distal to said tumor and peripheral blood lymphocytes.

7. A method for treating a patient identified by the method of claim 1, further comprising administering a therapy comprising delivering an effective amount an anti-EGFR IgG1 antibody or a biological equivalent thereof, to said patient.

8. The method of claim 7, wherein the anti-EGFR IgG1 antibody comprises Cetuximab or a biological equivalent thereof.

9. The method of claim 8 further comprising delivering an effective amount of a therapy selected from the group CPT-11, 5-fluorouracil (5-FU), LV, oxalplatinum and radiation therapy.

10. A method for identifying a patient expressing EGFR and responsive to anti-EGFR IgG1 antibody therapy or a biological equivalent of said therapy and wherein said patient suffers from a cancer treatable by inhibiting the EGFR pathway, comprising determining one or more genomic variant in a suitable sample selected from:

a) the allelic pattern for the FcγRIIa gene at amino acid position 131 and

b) the allelic pattern for the FcγRIIIa gene at amino acid position 158;

wherein the presence of at least one H at position 131 of FcγRIIa or at least one F at position 158 of FcγRIIIa, in said sample identifies the patient responsiveness to said therapy for said patient.

11. The method of claim 11, wherein the allelic pattern from said patient sample is H at position 131 of FcγRIIa and F at amino acid position 158 of FcγRIIIa.

12. The method of claim 10, wherein the cancer is selected from rectal cancer, colorectal cancer, metastatic colorectal cancer, colon cancer, gastric cancer, lung cancer, non-small cell lung cancer and esophageal cancer.

13. The method of claim 10, wherein the sample comprises tumor tissue, normal tissue adjacent to said tumor tissue, normal tissue distal to said tumor tissue and peripheral blood lymphocytes.

14. A method for identifying responsiveness to anti-EGFR therapy for a patient having cells expressing epidermal growth factor receptor (EGFR), comprising determining in a sample from said patient the allelic pattern selected from the FcγRIIa gene at position 131, wherein the presence of at least one H at position 131 of FcγRIIa in said sample identifies responsiveness to said therapy for said patient.

15. The method of claim 14, wherein the anti-EGFR therapy is anti-EGFR IgG 1 antibody therapy.

16. The method of claim 15, wherein the anti-EGFR antibody is Cetuximab.

17. The method of claim 14, wherein the cancer is selected from rectal cancer, colorectal cancer, metastatic colorectal cancer, colon cancer, gastric cancer, lung cancer, non-small cell lung cancer and esophageal cancer.

18. The method of claim 14, wherein the sample comprises tumor tissue, normal tissue adjacent to said tumor, normal tissue distal to said tumor and peripheral blood lymphocytes.

19. A method for treating a patient identified by the method of claim 14, further comprising administering a therapy comprising delivering an effective amount an anti-EGFR IgG1 antibody or a biological equivalent thereof, to said patient.

20. The method of claim 14, wherein the anti-EGFR IgG1 antibody comprises Cetuximab or a biological equivalent thereof.

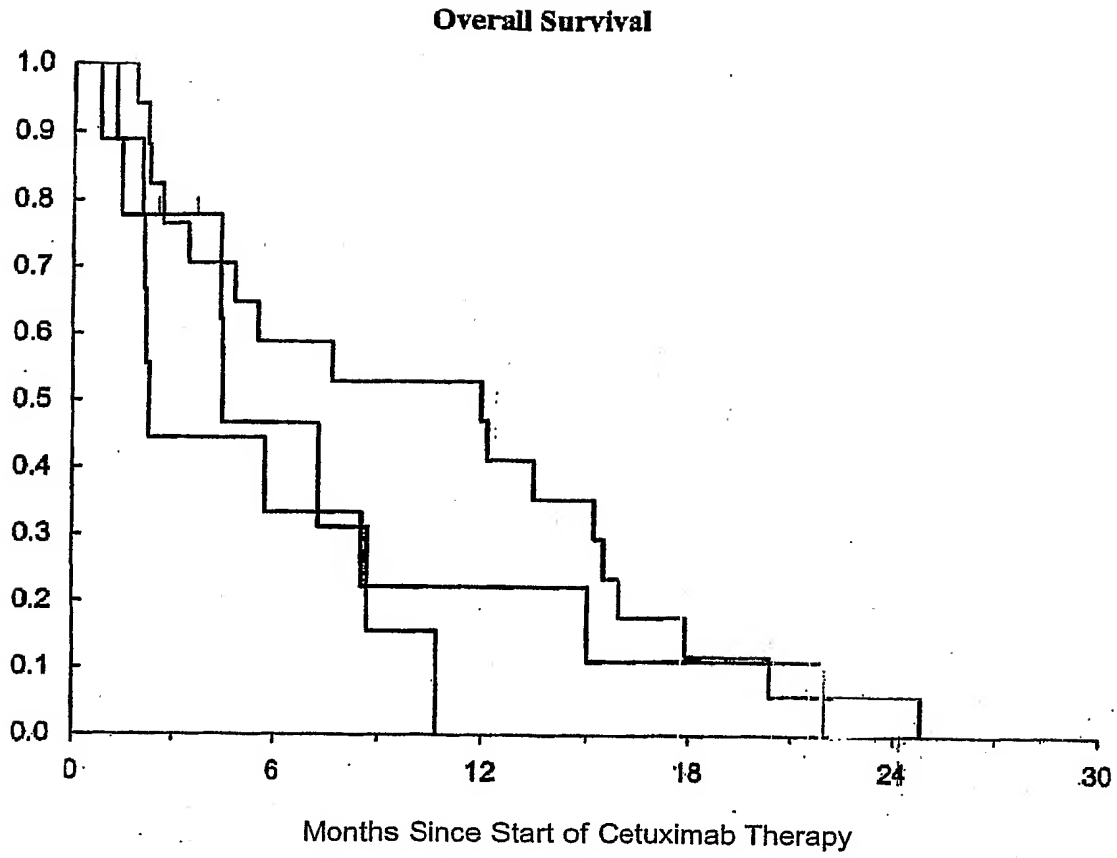
21. The method of claim 19, further comprising delivering an effective amount of a therapy selected from the group CPT-11, 5-fluorouracil (5-FU), LV, oxalplatinum and radiation therapy.

22. A method for identifying a patient expressing EGFR and responsive to anti-EGFR IgG1 antibody therapy or a biological equivalent of said therapy and wherein said patient suffers from a cancer treatable by inhibiting the EGFR pathway, comprising determining the allelic pattern for the FcγRIIa gene at amino acid position 131 wherein the presence of at least one H at position 131 of FcγRIIa identifies the patient responsiveness to said therapy for said patient.

23. The method of claim 22, wherein the allelic pattern from said patient sample is H/H at position 131.

24. The method of claim 22, wherein the cancer is selected from rectal cancer, colorectal cancer, metastatic colorectal cancer, colon cancer, gastric cancer, lung cancer, non-small cell lung cancer and esophageal cancer.

25. The method of claim 22, wherein the sample comprises tumor tissue, normal tissue adjacent to said tumor tissue, normal tissue distal to said tumor tissue and peripheral blood lymphocytes.


Overall Survival
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
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0
6
12
18
24
30
Months Since Start of Cetuximab Therapy

Figure 1

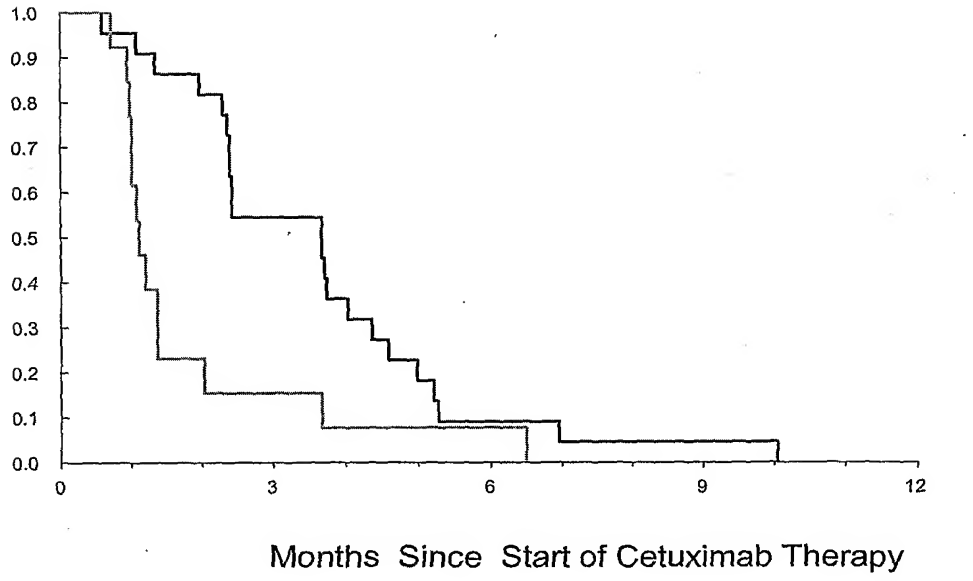
Estimated Probability of Being Progression-Free
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
3
6
9
12
Months Since Start of Cetuximab Therapy

Figure 2

Box No. VIII(v) DECLARATION: NON-PREJUDICIAL DISCLOSURES OR EXCEPTIONS TO LACK OF NOVELTY

The declaration must conform to the following standardized wording provided for in Section 215; see Notes to Boxes Nos. VIII, VIII(i) to (v) (in general) and the specific Notes to Box No. VIII (v). If this Box is not used, this sheet should not be included in the request.

Declaration as to non-prejudicial disclosures or exceptions to lack of novelty (Rules 4.17(v) and 51*bis*.1(a)(v)):

In relation to this international application

UNIVERSITY OF SOUTHERN CALIFORNIA declares that the subject matter claimed in this international application was disclosed as follows:

(i) Kind of disclosure: Publication

(ii) Date of publication: May 17, 2006

(iii) Title of disclosure: Abstracts to Meeting

(iv) Place of disclosure: not applicable

This declaration is made for the purposes of all designations.

☐ This declaration is continued on the following sheet, "Continuation of Box No. VIII (v)".

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US06/46127

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8): **C12Q 1/68**(2006.01);**C12P 19/34**(2006.01);**C07H 21/04**(2006.01);**A61K 39/00**(2006.01),**39/395**(2006.01)

USPC: 435/6,91.1,91.2;536/24.3;424/130.1,141.1,142.1,143.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6,91.1,91.2;536/24.3;424/130.1,141.1,142.1,143.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, EMBASE, BIOSIS, SCISEARCH, CAPLUS on STN

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WENG, W. Two Immunoglobulin G Fragment C Receptor Polymorphisms Independently Predict Response to Rituximab in Patients with Follicular Lympoma	1-2, 6, 10-11, 13-14, 18, 22-23, and 25
Y	CUNNINGHAM, D. Cetuximab Monotherapy and Cetuximab Plus Irinotecan in Irinotecan-Refractory Metastatic Colorectal Cancer 22 July 2004 (22.07.2004), N Engl J Med Vol. 354, No. 4, pages 337-345, especially page 337 and 343.	3-5, 7-9, 12, 15-17, 19-21 and 24

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 April 2007 (13.04.2007)	07 MAY 2007
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (571) 273-3201	Authorized officer Phuong Huynh Telephone No. (571) 272-1600

Form PCT/ISA/210 (second sheet) (April 2005)